

REMARKS

Claims 1-4 and 6-47 are currently pending in the application. Claims 10-11, 15, 18, 19, 23, 26, 27, 31, 34, 35, 39, 42, 43 and 47 are withdrawn. Claims 5 and 49-55 are cancelled. Claims 1-4, 6-9, 12-14, 16, 17, 20-22, 24, 25, 28-30, 32, 33, 36-38, 40, 41 and 44-46 are currently rejected. Claim 1 is herein amended. The amendment finds support in the specification and is discussed in the relevant sections below. No new matter is added.

Request for Reconsideration of Finality of Office Action

The Examiner made the Office Action dated December 7, 2005 final. Applicants respectfully request the Examiner withdraw the finality of the Office Action. MPEP 706.07 (a) states:

Under present practice, second or any subsequent actions on the merits shall be final, **except where the examiner introduces a new ground of rejection that is neither necessitated by applicant's amendment of the claims nor based on information submitted in an information disclosure statement** filed during the period set forth in 37 CFR 1.97(c) with the fee set forth in 37 CFR 1.17(p). [Emphasis added]

Applicants assert that the finality of the present Office Action is improper as the Examiner introduced two new grounds of rejections (112, first paragraph and 103) neither of which were necessitated by Applicants amendment to the claims, nor based on information submitted in an IDS. Thus, Applicants request the Examiner reconsider the finality of the present Office Action.

Priority

The Examiner notes that the Applicants had not filed a certified copy of the NZ336259 application as required by 35 U.S.C. 119(b). Filed herewith is a certified copy of the NZ336259. Applicants respectfully request the Examiner acknowledge the foreign priority claim.

Election or Restriction

The Examiner maintained the withdrawal of claims 15, 23, 31, 39 and 47. Applicants respectfully disagree with the Examiner for the same reasons set forth in the Applicants' response filed on October 4, 2005. The Examiner asserts that claims 15, 23, 31, 39 and 47 are

directed to HIF as a tumor restricted agent and that Applicants elected XAA. Applicants note that claims 15, 23, 31, 39 and 47 are also directed to XAA as being dependent on claims 1-4, and 6 respectively and that the election of XAA was a *species* election. Applicants assert that if claims 1-4, and 6 are allowed, dependent claims 15, 23, 31, 39 and 47 would also be free of the prior art and should be rejoined. In light of the above, Applicants request the Examiner rejoin claims 15, 23, 31, 39 and 47 if the claims these are dependent upon are allowed.

Rejections

35 U.S.C. § 112, first paragraph

Claims 1, 6-9 and 12-14 are rejected under 35 U.S.C. § 112, first paragraph. The Examiner believes that they are drawn to subject matter not described in the specification in a manner that enables one skilled in the art to make and use the invention. More specifically, the Examiner asserts that the specification lacks adequate disclosure with regards to teaching: (1) the administration of B7.1 **proteins** alone or in combination with DMXAA for the treatment of advanced or large tumors, and (2) that either a CAM (B7.1) protein or tumor growth-restricting agent alone would be ineffective in treating an advanced or large tumor burden. Specifically, the Examiner states:

Since the specification does not provide claimed method for treating a tumor with B7.1 protein with DMXAA for retarding or eradicating an advance or large tumor burden and since the specification does not provide enough evidence for each reagent having no effect when they are used alone for cancer treatment. One skilled in the art would not expect the claimed method will be successful in use the claimed method for treating cancer on the basis of teachings in the prior art or instant specification.

Applicants respectfully traverses these grounds for rejection and submits that the present specification satisfies the enablement requirement of 35 U.S.C. § 112, first paragraph. Enablement requires that the specification teaches one of ordinary skill in the art know how to make and use the invention. However, for enablement purposes, a specification need not teach what is well known in the art. *In re Wands*, 858 F.2d 731 (Fed. Cir. 1988). Moreover, some amount of experimentation is not fatal as long as the amount is not undue. *Id.*

One of ordinary skill in the art is enabled to practice the claimed invention without undue experimentation. The specification provides sufficient guidance to allow one having ordinary skill in the art to make and use a CAM protein (including a B7.1 protein) according to the claimed methods. The specification also teaches that a CAM molecule (B7.1) or tumor growth-restricting agent (DMXAA) alone does not eradicate an advanced or large tumor burden within the meaning of the claims.

CAM proteins (including B7.1) were known in the art at the time the present application was filed. In addition, the application teaches one how to make the CAM proteins (B7.1 protein) without undue experimentation. For example, paragraph 40 teaches the Genbank accession numbers and sources for numerous CAM molecules, including B7.1. Paragraph 9 teaches that the CAM proteins can be expressed by any vector available to the skilled artisan, including “expression plasmids such as pCDNA8 and pCDM8 and adenoviral and retroviral-based vectors (such as pLXSN and pLNCX).” Thus, the specification along with what was known in the art at the time of filing clearly enables one of ordinary skill to make the CAM proteins, including B7.1.

The specification details how to use a CAM (B7.1) **protein** with a tumor growth restricting agent (DMXAA) for the treatment of advanced or large tumors. While the working examples are directed to the administration of CAM (including B7.1) expression vectors, one of ordinary skill in the art would be able to practice the invention without undue experimentation by administering a CAM (B7.1) protein directly. The application teaches that the CAM proteins may be administered in lieu of the expression vectors:

Alternatively, the immunotherapeutic agent may be administered directly, in a form other than in a mammalian expression vector, that is, **it is not essential that the immunotherapeutic agent be administered using gene therapy.** For example, **T cell costimulatory CAM proteins that could be attached to the cell surface could be administered systemically.** [Paragraph 48, Emphasis added]

The application provides numerous teachings on how tumor restricting agents can be used in combination with CAMs. The specification includes detailed working examples describing methods of administering and testing tumor restricting agents in combination with CAMs and the results of these studies (See Figs. 1, 2, 4, 6 and 8).

Paragraph 76 and Fig. 4 teach a wide range of concentrations of the B7.1 expression vector that were effective at inducing an immune response that eradicated the tumors. Based on these studies one of skill in the art could identify an effective dose of a CAM protein (B7.1) without undue experimentation. Furthermore, the application teaches numerous methods for determining the effectiveness of CAM/tumor growth-restricting agent combination therapy. One of ordinary skill in the art would apply these teachings to practice the claimed invention without undue experimentation.

In addition to the teachings in the application, the administration of CAM proteins was known in the art at the time the application was filed. The specification need not teach what is well known in the art. *In re Wands*, 858 F.2d 731 (Fed. Cir. 1988).

Methods of administering CAM proteins (e.g., B7.1) were known in the art at the time of filing (See exhibits A-D). McHugh et al. (Exhibit A) teaches the making and use of GPI-B7.1 proteins in tumor immunotherapy. (See abstract and subsections under Materials and Methods titled, Preparation of Tumor Membranes and Protein Transfer of GPI-B7-1 and Immunization of Mice and Tumor Protection Studies). Dunussi-Joannopoulos et al. (Exhibit B) teaches the immunization of mice with B7.1 transduced AML cells (p. 2916, second column, 6th paragraph). Sturmhoefel et al. (Exhibit C) teaches the making and use of fusion proteins having the extracellular portion of B7.1 or B7.2 and the Fc portion of IgG2a in vaccines. (See abstract, and subsections under Materials and Methods titled, B7-IgG Fusion proteins and Vaccination Protocols). Moro et al. (Exhibit D) teaches the making and use of biotinylated recombinant soluble B7-1 and B7-2 immunoglobulin G molecules in tumor immunity. (See abstract and subsections of Materials and Methods titled, Generation of Recombinant Soluble B7-Ig, Preparation of bio-19E12mAb and bio-B7-IgG and In Vivo Studies.) Thus, at the time of filing methods of making and administering CAM proteins (e.g., B7.1) were well known in the art. In light of the above, one would be able to make and use CAM proteins (B7.1) according to the claimed method without undue experimentation.

The Examiner also asserts that the application does not teach one how to perform the claimed method when either reagent alone (e.g., CAM or DMXAA) would be ineffective in retarding or eradicating an advanced or large tumor burden.

Applicants respectfully disagree with the Examiner. First, Applicants note that claim 1 is herein amended to state, “either of which alone would be ineffective in ~~retarding or~~ eradicating an advanced or large tumor burden.”

Throughout the application there are numerous teachings that illustrate that monotherapy is ineffective in treating advanced or large tumors but that combination therapy is effective (See Figures. 1, 2 and 10). For example, paragraph 74 referring to Fig. 1 teaches that CAM monotherapy is ineffective for treating large tumors:

CAM Gene Monotherapy is Unable to Check the Growth of Large Tumours. . . . In contrast, as shown in FIG. 1a, **larger tumours (>0.5 cm) are refractory to treatment in response to B7.1** and several other costimulatory CAMs. Tumour growth is slowed, but ultimately the tumour grows unchecked. [Emphasis added]

Paragraph 75 teaches the same is true for DMXAA and FAA monotherapy:

DMXAA and FAA are Unable to Check the Growth of Large Tumours. Systemic administration of optimal doses of DMXAA and FAA to mice bearing large EL-4 tumours (0.6-0.8 cm in diameter) led to immediate reductions in the sizes of tumours (FIG. 1b), accompanied by marked tumour necrosis (refer below). DMXAA was the more potent of the two reagents, causing tumors to shrink to 0.1-0.2 cm over a period of 3 weeks, whereas the tumours of FAA-treated animals were reduced to 0.2-0.4 cm in diameter. **However, tumours began to grow unchecked by day 28 and animals had to be sacrificed during the sixth week.** [Emphasis added]

Thus, monotherapy with a CAM (e.g., B7.1), DMXAA and FAA are unable to eradicate large tumors. However, combination therapy utilizing CAM (e.g., B7.1) and DMXAA is effective. For, example paragraph 76 states:

Combined Therapy by Timed Delivery of B7.1 and DMXAA/FAA Eradicates Large Tumours. We hypothesized that simultaneous administration of the B7.1 pCDM8 expression vector and DMXAA/FAA might impair CAM-mediated anti-tumour immunity, as dying and necrotic

tumour cells would not be able to adequately express B7.1. This notion proved correct, and hence established tumours (0.6-0.8 cm in diameter) were first treated with B7.1 to stimulate anti-tumour immunity, and DMXAA and FAA were administered one day later to retard tumour growth. **Remarkably, tumours rapidly diminished in response to the combination of B7.1 and DMXAA accompanied by massive necrosis, such that by the third week of treatment tumours had completely disappeared (FIG. 1b).** [Emphasis added]

Thus, the specification clearly teaches that monotherapy was unable to eradicate an advanced or large tumor burden while combination therapy was effective. In light of the above arguments and amendments, Applicants respectfully request the Examiner withdraw the 35 U.S.C. §112, first paragraph rejection of claims 1, 6-9 and 12-14.

35 U.S.C. § 103

The Examiner rejected claims 2-4, 16, 17, 20-22, 24, 25, 28-30, 32, 33, 36-38, 40, 41, 45 and 46 as unpatentable under 35 U.S.C. 103(a) over Futami et al. in view of Olsson et al. The Examiner asserts that Futami et al. teaches methods of treating a tumor with analogues of XAA in combination with IL-2. The Examiner asserts Olsson et al. teaches that CD80 (B7.1) induces IL-2. Based on these teachings the Examiner concludes that:

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the claimed invention was made to use the method to eradicate any advanced or large tumor by administering analogs of XAA in combination of CAM comprising CD80 with the expected result for cancer treatment. . . . One of ordinary skill in the art would have been motivated with a reasonable expectation of success to combine the teachings of Olsson et al., to the teaching of Futami et al., to treat cancer by combining CAM and analogs of XAA comprising DXMAA because Futami et al. have shown that IL-2 and analogues of XAA have a synergy effect when they are used for cancer therapy and Olssone et al., have shown IL-2 can be induced by a CAM, CD80.

Applicants respectfully traverse the rejection.

For the reasons described below, the Examiner has failed to establish a *prima facie* case of obviousness under the requirements of 35 U.S.C. § 103(a). To establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of

ordinary skill in the art, to modify the reference or to combine reference teachings (*In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991)). Second, there must be a reasonable expectation of success. *Id.* The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on Applicants' disclosure. *Id.* Finally, the prior art reference (or references when combined) must teach or suggest *all the claim limitations*. *In re Royka*, 490 F.2d 981, 180 U.S.P.Q. 580 (C.C.P.A. 1974).

The prior art references fail to provide some suggestion or motivation to modify the references or to combine the references teachings. The Federal Circuit has stated that "[o]bviousness cannot be established by combining the teachings of the prior art to produce the claimed invention, absent some teaching[,] suggestion or incentive supporting the combination" (*In re Geiger*, 815 F.2d 686, 688, 2 U.S.P.Q.2d 1276, 1278 (Fed. Cir. 1987)) and that "[i]t is impermissible . . . simply to engage in a hindsight reconstruction of the claimed invention, using the applicant's structure as a template and selecting elements from references to fill the gaps. . . . The references themselves must provide some teaching whereby the applicant's combination would have been obvious." (*In re Gorman*, 18 U.S.P.Q.2d 1885, 1888 (Fed. Cir. 1991)).

There is no teaching or suggestion in either reference that treatment of XAA and a CAM (B7.1) will produce a synergistic effect in eradicating advanced or large tumors. Futami et al. does not even teach a CAM molecule. Furthermore, Olsson et al. only teaches that B7.1 signaling may induce IL-2 production. Neither reference provides any motivation to combine.

The Examiner appears to engage the hindsight conclusion that because B7.1 induces IL-2 one would be motivated to replace the IL-2 of Futami et al. with B7.1 for the treatment of cancer. Applicants find the Examiners rational strained. Applicants assert that there is no motivation to replace the administration IL-2 of Futami et al. with the administration of a CAM protein (e.g., B7.1) to get IL-2 production.

Furthermore, all claims are directed to a method of eradicating an **advanced or large tumor**. There is no mention or suggestion in either references asserted by the Examiner that

large tumors could be treated successfully by using a combination of a tumor growth-restricting agent (e.g., XAA derivative) and B7.1.

Even when the references are combined they fail to teach or suggest all the claim limitations. The present claims are generally directed to methods for treating advanced or large tumors by administering a CAM protein (B7.1) in combination with a tumor growth-restricting agent (DMXAA). CAM and tumor growth restricting agents have been shown by Applicants to have unexpected synergistic properties which eradicate advanced or large tumors.

When both references are combined the references fail to teach; (1) **administering a CAM protein (B7.1)** to a patient (2) for eradicating an **advanced or large tumor**. There is no teaching within Futami et al. or Olsson et al. for administering a CAM protein (B7.1), let alone administering a CAM protein (B7.1) in combination with XAA. Furthermore, there is no teaching in either reference for using a CAM protein (B7.1) to treat cancer, let alone for the treatment of advanced or large tumors. Thus, for these reasons the prior art references fail to teach all elements of the claims.

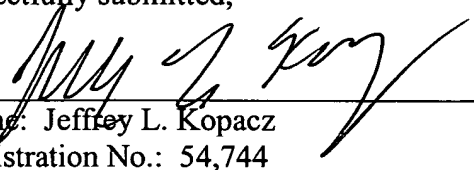
Neither Futami et al. nor Olsson et al. suggest a reasonable expectation of success in combination treatment utilizing XAA and a CAM protein (e.g., B7.1) for eradicating advanced or large tumors. This is evidenced by the fact that Olsson et al. teaches that the effect of B7.1 are governed through the proliferation of CD4 cells which are then induced by B7.1 to release IL-2. However, in the present invention it is shown that blocking the effect of CD4 cells did not inhibit the efficacy of the claimed treatment combination (see paragraph 80, and figure 3). The application explicitly states that the most important mechanism of action for the combination is governed by CD8 cells (see paragraph 23). Applicants assert that the based on the cited prior art there is no reasonable expectation of success.

Thus given the above, those skilled in the art at the time of filing of the present application would not have expected a combination of a tumor growth-restricting agent and a CAM (B7.1) to provide a synergistic effect in the treatment of advanced or large tumors. In light of the above, Applicants respectfully request the Examiner withdraw the rejection of claims 2-4, 16, 17, 20-22, 24, 25, 28-30, 32, 33, 36-38, 40, 41, 45 and 46 under 35 U.S.C. §103(a).

Applicants submit that in view of the foregoing remarks, all issues relevant to patentability raised in the Office Action have been addressed. Applicants respectfully request the withdrawal of rejections over the claims of the present invention.

Respectfully submitted,

Date: June 7, 2006


Name: Jeffrey L. Kopacz

Registration No.: 54,744

Customer No.: 29933

Edwards Angell Palmer & Dodge LLP

P.O. Box 55874

Boston, MA 02205

Tel.: (617) 239-0100



Protein Transfer of Glycosyl-phosphatidylinositol-B7-1 into Tumor Cell Membranes: A Novel Approach to Tumor Immunotherapy¹

Rebecca S. McHugh, Shanmugam Nagarajan, Yi-Chong Wang, Kenneth W. Sell, and Periasamy Selvaraj²

Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, Georgia 30322

ABSTRACT

Modification of tumor cells with one or more costimulatory adhesion molecules has been proposed as a means to develop therapeutic cancer vaccines for use in human immunotherapy. Expression of B7-1 (CD80) in tumors by gene transfer creates an immunogenic tumor cell that induces antitumor immunity and protects mice from further challenge with wild-type tumor cells. In this report, we demonstrate that protein transfer of glycosyl-phosphatidylinositol (GPI)-anchored costimulatory molecules into tumor cell membranes could be used as an alternative to gene transfer for tumor immunotherapy. Incubation of isolated tumor membranes with purified GPI-anchored B7-1 results in stable incorporation of B7-1 on tumor cell membranes within a few hours. Immunization of C57BL/6 mice with EG7 tumor membranes modified to express GPI-B7-1 by protein transfer induces tumor-specific T-cell proliferation and CTLs. In addition, immunization with these EG7 membranes protects mice from parental tumor challenge. The protein transfer approach used here does not require foreign vectors or live tumor cells and is completed within a matter of hours. Irradiated cells or membrane preparations from fresh or frozen tumor tissue can be used. Therefore, protein transfer of glycolipid-anchored molecules provides an efficient and novel approach to modify tumor membranes for human immunotherapy. This approach is not limited to costimulatory molecules because any cell surface protein can be converted to a GPI-anchored form by recombinant techniques.

INTRODUCTION

For T cells to proliferate and respond to an antigen, two signals are required (1). Engagement of the T-cell receptor with antigen-MHC complexes provides the initial signal. The second signal, termed costimulation, is received from any number of adhesion receptor-ligand interactions between the antigen-presenting cell and T cell, such as B7/CD28 (2-4). The absence of costimulation during T-cell recognition of a specific antigen results in T-cell unresponsiveness (5, 6). Many tumor cells express antigen-MHC complexes while failing to express appropriate cell adhesion molecules necessary for T-cell costimulation (7-11). By lacking these costimulatory adhesion molecules, tumor cells may thus escape host immunity.

Expression of costimulatory adhesion molecules such as B7-1, B7-2, or intercellular adhesion molecule 1 on tumor surfaces by gene transfer techniques can create a more immunogenic tumor cell that induces protective immunity against a parental tumor challenge (7-11). Recently, we (12) and others (13) have shown that costimulatory molecules can be expressed on the surface of cells by another method, protein transfer. This method uses proteins that are anchored to the membrane through GPI.³ These proteins do not span the membrane but have a lipid tail that anchors the protein to the outer leaflet of the lipid bilayer. Given this unique attachment, these proteins can spontaneously incorporate into amphiphilic surfaces such as cell mem-

branes (14, 15). Using the EG7 tumor system, we demonstrate herein that EG7 tumor cell membrane preparations modified to express GPI-B7-1 can stimulate an antitumor immune response and protect mice from a challenge with live wild-type tumor cells.

MATERIALS AND METHODS

Animals and Cell Culture. Female C57BL/6 mice were purchased from Jackson Laboratories. EG7 tumor cells were maintained in DMEM supplemented with 10% FCS, 400 µg/ml Geneticin, 50 µg/ml gentamycin, and 2 mM glutamine. EG7 cells are derived from the murine T-cell lymphoma EL4 transfected with cDNA for ovalbumin and lack B7-1 expression (16). Although C57BL/6 mice have a repertoire of T cells that are specific for the ovalbumin CTL epitope presented by MHC class I, EG7 cells still form solid tumors in mice and fail to provide protective immunity (17).

Preparation of Tumor Membranes and Protein Transfer of GPI-B7-1. Membranes were prepared as described by Maeda *et al.* (18). Briefly, cell pellets were homogenized on ice in solubilization buffer [20 mM Tris (pH 8.0) containing 10 mM NaCl, 0.1 mM MgCl₂, 0.02% NaN₃, and 0.1 mM phenylmethylsulfonyl fluoride] and ultracentrifuged (93,000 × g) for 1 h over a 41% sucrose gradient. The interface was recovered and washed three times in solubilization buffer by centrifugation.

For incorporation of GPI-B7-1, frozen (-80°C) or fresh membranes were resuspended to 100 µl using serum-free RPMI 1640 containing 100 µg/ml ovalbumin. Purified GPI-B7-1 (10 µg/ml) was added to the membranes, and the mixtures were shaken for 4 h at 37°C in siliconized microfuge tubes. Nonincorporated control EG7 membranes were incubated at 37°C in the same buffer without GPI-B7-1. The membranes were washed two more times and either analyzed by ELISA or resuspended in HBSS or HBSS containing 2 ng of rIL-12, using a 20-gauge needle for mice immunizations. The GPI-anchored form of FcγR III, CD16B, was also used to modify tumor cell membranes by protein transfer. CD16B was immunoaffinity-purified from CD16B-transfected K562 cells and diluted in the above-mentioned buffer to a final concentration of 20 µg/ml for incubation of the membranes as described. For ELISA, the membranes were coated onto microtiter plate wells overnight at 4°C. The wells were blocked with complete RPMI1640 containing 10% FCS, and then the membranes were analyzed using X63 (negative control), PSRM-3 (antihuman B7-1 mAb), M1/42 (antimouse monomorphic class I), or CLBFCgran-1 (anti-CD16). The absorbance of PSRM-3-binding wells was normalized to the absorbance of M1/42-binding wells, which was designated as 1.0.

Immunization of Mice and Tumor Protection Studies. C57BL/6 mice (five mice/group) were immunized i.p. with 100 µl of HBSS, EG7 membranes (100 µg of equivalent protein), or GPI-B7-1-incorporated EG7 membranes twice at a 2-week interval. Three weeks after the final immunization, the spleens were harvested, and T cells were purified using mouse T-cell enrichment columns (R&D Systems) to 95% purity as analyzed by flow cytometry. The T cells were used in either a MLTR or CTL assays.

Other C57BL/6 mice (five mice/group) were immunized as described above, except for the addition of rIL-12 (PharMingen, San Diego, CA) treatments *in vivo*. rIL-12 (2 ng/mouse) was administered i.p. beginning 1 week after the first immunization. This treatment was continued every 4 days for 2 weeks. Three weeks after the final membrane immunization, T cells were purified as described and used in CTL assays.

For tumor challenge experiments, mice were immunized s.c. in the hind flank with HBSS, EG7 membranes, or GPI-B7-1-incorporated EG7 membranes, with or without 2 ng of rIL-12. Two weeks later, the mice were boosted. EG7 cells (10⁵) were injected s.c. at a remote site 1 week after the boost. Mice were monitored daily for tumor growth and euthanized when tumors reached 2 cm in diameter.

Received 7/21/97; accepted 3/17/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Supported by NIH Grant CA70833.

² To whom requests for reprints should be addressed, at Department of Pathology, 7307 Woodruff Memorial Building, Emory University School of Medicine, Atlanta, GA 30322.

³ The abbreviations used are: GPI, glycosyl-phosphatidylinositol; IL, interleukin; rIL, recombinant IL; mAb, monoclonal antibody; MLTR, mixed lymphocyte-tumor reaction.

T-Cell Proliferation and CTL Assays. For the MLTR, T cells (10^5) purified from immunized mice were cocultured with irradiated (10,000 rads) EG7 cells (2×10^4) for 5 days in a 5% CO₂ incubator at 37°C. T-cell proliferation was measured by pulsing the wells with 1 μ Ci of [³H]thymidine for the last 18 h of culture.

For CTL assays, T cells were restimulated *in vitro* for 5 days with irradiated (10,000 rads) EG7 cells. On the second day of the restimulation, 10 units/ml rIL-2 (PharMingen) were added. Live T cells were recovered by density sedimentation using Histopaque 1077 (Sigma) and resuspended to 10^7 cells/ml. EG7 and concanavalin A blasts (splenocytes treated with 10 ng/ml concanavalin A for 3 days) were labeled with 200 μ Ci of ⁵¹Cr (Amersham, Arlington Heights, IL) for 2 h at 37°C. These cells were washed and resuspended to 10^5 cells/ml. The effectors and targets were mixed at various ratios, and a standard 4-h ⁵¹Cr release assay was performed.

For T-cell depletion, T cells were pretreated with either 53.6 (anti-CD8) or 145-2C11 (anti-CD3) for 30 min. The coated cells were then incubated at 37°C for 30 min with the rabbit complement. Live cells were recovered as described above and used in the ⁵¹Cr release assay.

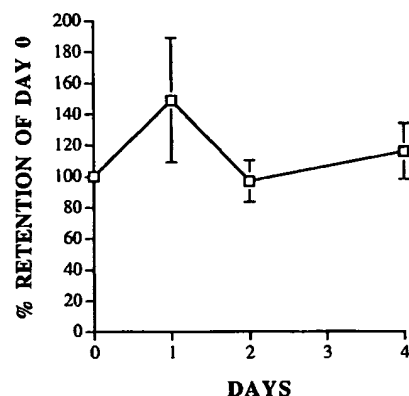
RESULTS AND DISCUSSION

Cell membranes were prepared from EG7 cells (18) and modified to express GPI-B7-1 by protein transfer. GPI-B7-1 was stably incorporated in these membranes after incubation with purified GPI-B7-1 for 4 h at 37°C (Fig. 1A). These membranes were analyzed for B7-1 and MHC class I expression by ELISA. After incubation with 10 μ g/ml purified GPI-B7-1, we obtained GPI-B7-1 expression at 50% of MHC class I expression (data not shown). Nearly 100% of the incorporated GPI-B7-1 was retained in the membranes for at least 4 days under culture conditions (Fig. 1A). To determine whether the GPI-B7-1-incorporated membranes could stimulate tumor-specific immune responses *in vivo*, we first measured the proliferation of T cells from mice immunized with either HBSS, EG7 membranes, or GPI-B7-1-incorporated EG7 membranes in a MLTR. As seen in Fig. 1B, T cells from mice immunized with GPI-B7-1-incorporated EG7 membranes proliferated fivefold over the background when cocultured with wild-type EG7 cells. T cells from the HBSS control- and buffer-treated EG7 membrane-primed mice were unable to mount a significant proliferative response to EG7 tumor cells.

Next, the ability to generate a CTL response against the parental tumor was investigated. After immunization, T cells were purified and restimulated *in vitro* with irradiated EG7 cells for 5 days and then assayed for cytotoxicity to EG7 cells. T cells from mice primed with GPI-B7-1-incorporated EG7 membranes had an increased cytotoxic response to the EG7 targets in comparison to the EG7 membranes or HBSS-immunized controls (Fig. 2A). Analysis of CTL activity from two independent experiments shows an average of 14% specific lysis of target cells at the 50:1 E:T ratio.

Many cytokines have been shown to augment antitumor immune response (19). In particular, IL-12 has been reported to augment the production of cytotoxic cells and work in concert with B7-1 in generating antitumor immunity (20, 21). Therefore, we investigated whether IL-12 could further enhance the CTL activity induced by tumor membranes modified with GPI-B7-1. IL-12 was administered i.p. at regular intervals during the membrane immunizations. As shown in Fig. 2B, IL-12 administration increased the specific lysis of the EG7 targets to 54% by the T cells primed with GPI-B7-1 reconstituted EG7 membranes as compared to 12.4% specific lysis from T cells of mice immunized with the same membranes in the absence of IL-12 treatment. IL-12 treatment of mice immunized with HBSS or EG7 membranes did not enhance CTL activity. In this assay, another GPI-B7-1 molecule constructed using the GPI signal sequence from LFA-3 gave a similar response, indicating that the origin of the GPI anchor signal sequence does not influence B7-1 function (data not shown).

A



B

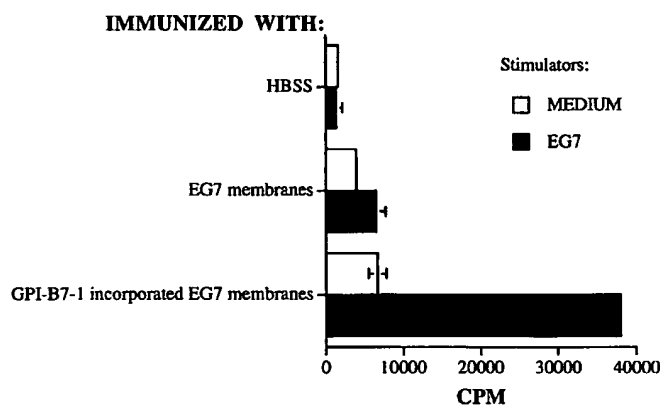


Fig. 1. Immunization with GPI-B7-1-incorporated EG7 membranes induces a T-cell-proliferative response to the parental tumor. **A**, GPI-B7-1 expressed by protein transfer is stable on EG7 membranes. EG7 tumor membranes were prepared as described previously (18). Approximately 200 μ g of EG7 membrane protein equivalent (determined using the DC protein assay; Bio-Rad) were resuspended in 300 μ l of buffer (RPMI1640 containing 5 mM EDTA and 100 μ g/ml ovalbumin) and 10 μ g/ml purified GPI-B7-1. The membranes were incubated for 4 h at 37°C in siliconized tubes. After incubation, membranes were washed, and an aliquot was assayed for the incorporation of GPI-B7-1 by ELISA (day 0). The remaining membranes were resuspended in PBS containing 1% FCS and incubated at 37°C for up to 4 days in a CO₂ incubator. At various time points, aliquots of the membranes were washed and assayed for GPI-B7-1 expression by ELISA. Membrane proteins were detected by ELISA using P5RM-3 (anti-B7-1 mAb) and M1/42 (anti-MHC class I mAb). The expression of GPI-B7-1 at the indicated time points is represented as the percentage of day 0 incorporation. Data points are the mean \pm SD from three experiments. **B**, immunization with GPI-B7-1-incorporated EG7 membranes induces a T-cell-proliferative response to the parental tumor. Experiments with mice were performed according to Emory University guidelines. C57BL/6 female mice were immunized i.p. with either HBSS or 100 μ g of EG7 membranes protein equivalent incubated in the above-mentioned buffer with or without GPI-B7-1. After 2 weeks, the mice received a second i.p. injection of HBSS or of the different membrane preparations. Three weeks later, the spleens were harvested, and T cells were enriched by T-cell purification columns (R&D Systems). T cells (10^5 cells/well) were cocultured with 2×10^4 irradiated (10,000 rads) EG7 cells/well for 5 days. All wells were pulsed with 1 μ Ci of [³H]thymidine for the last 18 h of culture. These results are representative of two independent experiments.

To confirm that it is the specific incorporation of GPI-B7-1 and not the addition of any lipid-modified protein that confers immunogenicity, tumor membranes incorporated with CD16B, the naturally GPI-anchored form of Fc γ R III (22), were included as a control. As shown in Fig. 2C, no CTL responses were observed in mice immunized with CD16B-incorporated EG7 membranes, even with the coadministration of IL-12.

To determine the nature of the effector cells mediating antitumor cytotoxicity, CTL responses were analyzed after the depletion of CD8⁺ T cells *in vitro*. Treatment of effector cells with anti-CD8

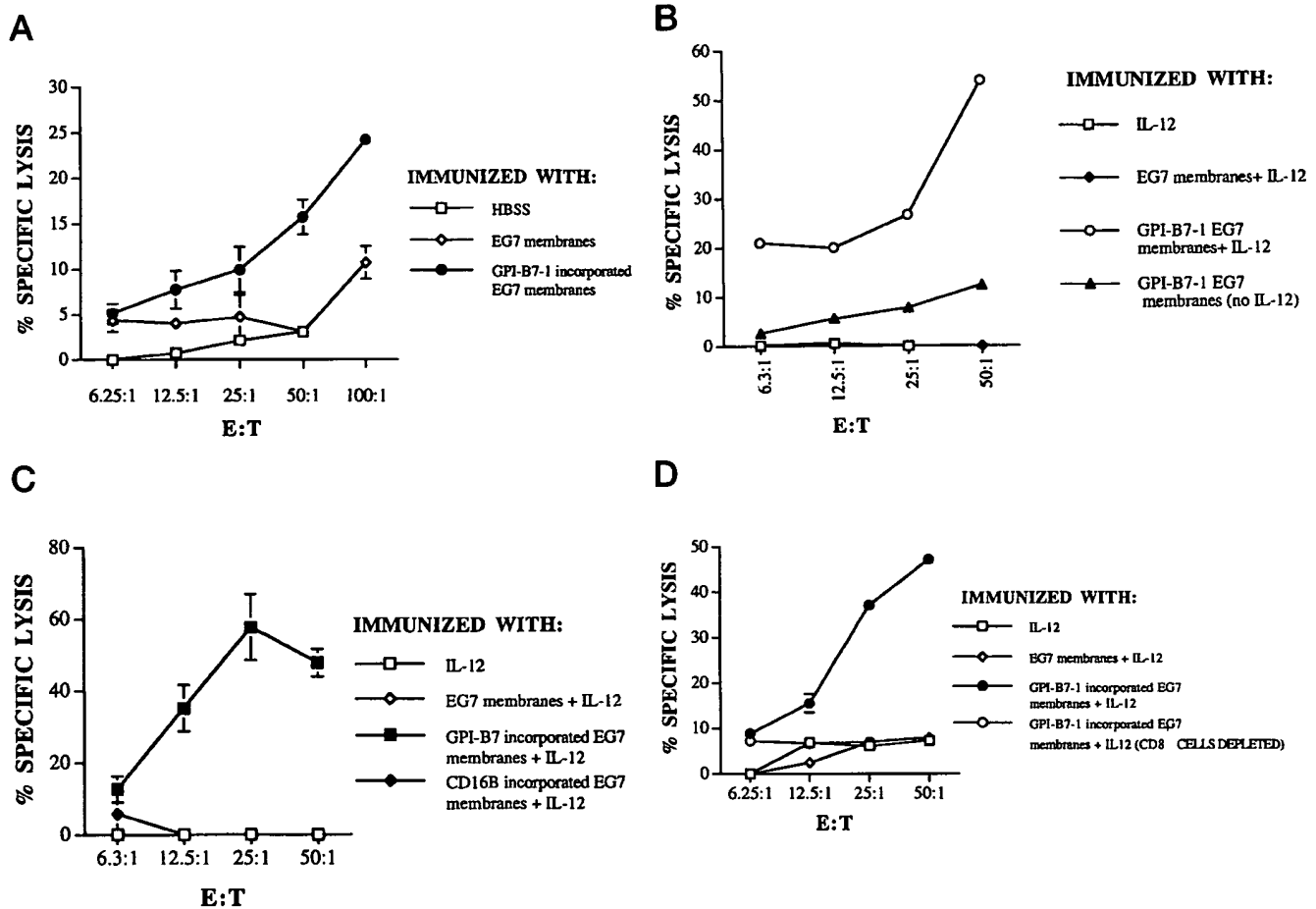


Fig. 2. *A*, immunization with GPI-B7-1-incorporated membranes induces CTL activity toward the parental tumor. Mice were immunized with either HBSS (□), buffer-treated EG7 membranes (◇), or EG7 membranes incorporated with GPI-B7-1 (●) as described in Fig. 1*B*. After the immunizations, T cells were purified and restimulated *in vitro* with irradiated EG7 cells for 5 days. Live cells were recovered and used as effector cells at various E:T ratios in a standard 4-h ⁵¹Cr release assay with EG7 targets. *B*, enhancement of GPI-B7-1 EG7 membrane-induced CTL activity by IL-12. C57BL/6 mice were immunized as described in *A* with the addition of IL-12 *in vivo*. One week after the first immunization, mice were treated with 2 ng of rIL-12 i.p. This treatment was continued every 4 days for 2 weeks. The T cells obtained from mice immunized with IL-12 (□), buffer-treated EG7 membranes + IL-12 (◇), EG7 membranes incorporated with GPI-B7-1 + IL-12 (○), and EG7 membranes incorporated with GPI-B7-1 without IL-12 (▲), were used in a ⁵¹Cr release assay. The data points are the average of triplicates, and the SDs were less than 1% of the mean. *C*, modification of EG7 membranes with GPI-CD16B did not induce tumor-specific CTLs. The immunization protocol was the same as that described in *B*, except that one group of mice was immunized with EG7 membranes that were incorporated with CD16B (GPI-anchored FcγR III) using the protocol described in Fig. 1*A*. CTL activity against EG7 cells of T cells recovered from mice immunized with IL-12 (□), buffer-treated EG7 membranes + IL-12 (◇), GPI-B7-1-incorporated EG7 membranes + IL-12 (■), or CD16B-incorporated EG7 membranes + IL-12 (●), was measured. *D*, depletion of CD8⁺ cells abrogates CTL activity induced by GPI-B7-1-modified EG7 membranes and IL-12. Mice were immunized with IL-12 (□), buffer-treated EG7 membranes + IL-12 (◇), or GPI-B7-1-incorporated EG7 membranes + IL-12 (●). T cells were restimulated *in vitro* and used in a 4-h ⁵¹Cr release assay as described in *A*. An aliquot of the effector cells obtained from mice immunized with EG7 membranes incorporated with GPI-B7-1 + IL-12 was depleted of CD8⁺ cells by treatment with 53.6 (anti-CD8) mAb and the rabbit complement. The live cells (○) were then recovered and used as effector cells in the same assay. All assays are an average of triplicate wells ± SDs.

antibody and complement eliminated nearly 83% of the cytotoxic activity, indicating that CD8⁺ cells are the major effector of cytotoxicity (Fig. 2*D*). Similarly, the *in vitro* depletion of CD3⁺ T cells from mice primed with GPI-B7-1 reconstituted membranes completely eliminated cytotoxicity (data not shown), indicating that T cells and not natural killer cells were the effector of the immune response to EG7 tumor cells.

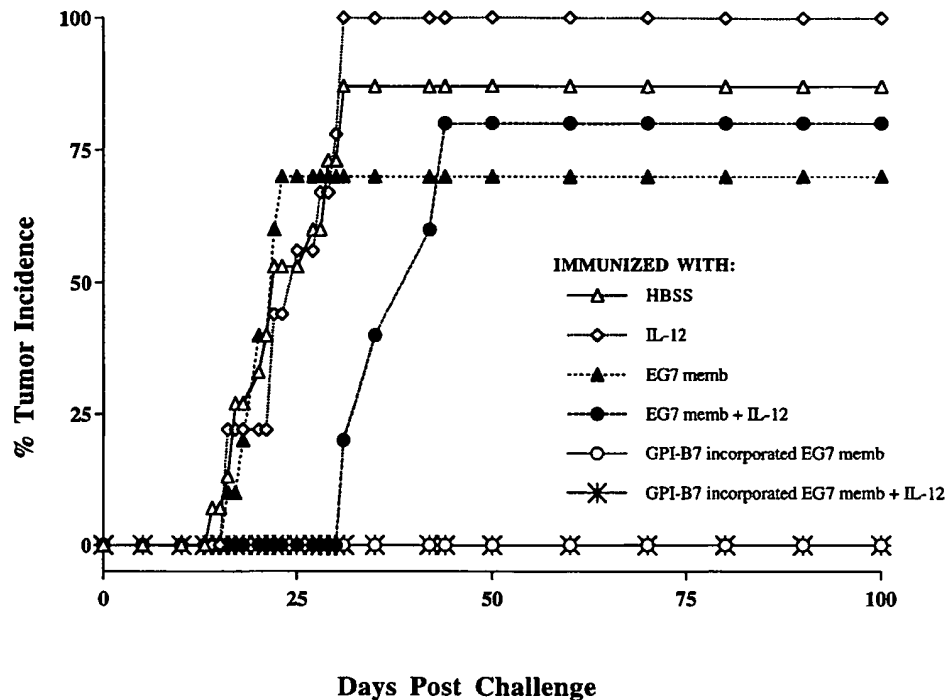
Although we have shown that EG7 membranes modified with GPI-B7-1 can generate CTLs against the parental tumor, it is necessary to determine whether these membrane preparations can immunize and protect mice against a subsequent challenge with the parental tumor. To determine this, mice were immunized twice s.c. in the hind flank with GPI-B7-1-modified or buffer-treated membranes in the presence or absence of IL-12. One week after the final immunization, mice were challenged s.c. with wild-type EG7 cells. After a few weeks, tumors developed and grew rapidly in mice immunized with HBSS, IL-12, or buffer-treated EG7 membranes (Fig. 3). In contrast, mice immunized with EG7 membranes modified with GPI-B7-1, with

or without coinjections of IL-12, remained tumor free for the duration of the experiment.

As shown in Fig. 3, only mice immunized with GPI-B7-1-modified EG7 membranes were protected from the tumor challenge. Although tumors grew in mice immunized with EG7 membranes and IL-12, there was a delay in tumor growth (Fig. 3). This was reproducible, suggesting that IL-12 treatment may enhance immune activity to unmodified EG7 membranes. However, tumors still developed, indicating that immunization with EG7 membranes and IL-12 was unable to induce a sufficient antitumor immune response. The ovalbumin used during GPI protein transfer did not influence the immunity against tumors because mice immunized with tumors modified in presence or absence of ovalbumin showed a similar level of protection against tumor challenge (data not shown). These results demonstrate that EG7 membranes modified to express B7-1 by protein transfer can effectively induce antitumor immunity and protect mice from further tumor challenge.

Protein transfer techniques can also be used to introduce B7-1 onto

Fig. 3. Immunization with GPI-B7-1-incorporated membranes provided protection from the parental tumor *in vivo*. C57BL/6 mice (four to five mice/group) were immunized with either HBSS (Δ), 2 ng of IL-12 (\diamond), 100 μ g of the protein equivalent of buffer-treated EG7 membranes with (\bullet) or without (\blacktriangle) 2 ng of IL-12, or 100 μ g of the protein equivalent of GPI-B7-1-incorporated EG7 membranes with (*asterisk*) or without (\circ) 2 ng of IL-12 s.c. in the right flank. After 2 weeks, the mice were boosted with the same material. One week later, mice were challenged s.c. with 5×10^6 EG7 cells in the opposite side and then monitored daily for tumor incidence. Two independent experiments of four to five mice/group were conducted. The data points are compiled from the results obtained from a total of 10 mice in each experimental group, with the exception of the IL-12 group, which had 9 mice.



the surface of intact live tumor cells. We have observed, however, that intact live cells lose surface expression of GPI-B7-1 quickly under culture conditions (12). This is most likely due to the fact that the GPI-anchored B7-1 molecule is exogenously added to the membrane and therefore cannot be replaced after cell division, internalization, or shedding. Therefore, expression on live cells will be lost rather quickly. Live tumor cells, however, are an unlikely candidate for administration to human patients. Other preparations of tumor cells would need to be used, such as irradiated cells or cell membrane preparations. Preliminary studies in our laboratory on tumor cell lines such as P815, a murine mastocytoma, and K1735, a murine melanoma, show that immunization with GPI-B7-1-incorporated γ -irradiated tumor cells can induce a tumor-specific T-cell-proliferative response in mice.⁴ However, in humans, irradiated tumor cells may cause problems if not all cells have been killed by irradiation. In addition, some murine tumor cells, when irradiated, have been shown to be poor inducers of an immune response toward the parental tumor (23, 24).

As an alternative, we have used isolated tumor cell membranes. These preparations offer many advantages because they do not divide or have the metabolic functions of cells and can therefore provide a stable environment for incorporated GPI-anchored molecules. Membranes can also be easily stored in frozen aliquots or freshly prepared from frozen tumor tissue. These membranes can then be quickly incorporated with GPI-anchored proteins for convenient immunization protocols. Membrane preparations also retain the ability to interact with and stimulate cells in culture. Membranes of Chinese hamster ovary cells expressing GPI-B7-1 can polyclonally stimulate T cells in the presence of suboptimal doses of phorbol 12-myristate 13-acetate (data not shown). In other studies, membranes from T helper cells have been shown to stimulate B cells *in vitro* (25, 26).

Currently, gene transfer is the method of choice for the expression of new proteins in cells. However, gene transfer may present problems for human tumor immunotherapy in the clinical setting. This method

introduces foreign vectors, some of which are of viral origin. At the site of incorporation, these vectors could introduce chromosomal mutations. Also, due to the immunity developed against vaccinia viral proteins, the vaccinia-based vectors can be used only once to deliver the desired genes (27). Other viral vectors, such as adenovirus, also increase cellular infiltration at the site of delivery, indicating an immune response to the vector that would prevent its subsequent use for gene therapy (28, 29). The protein transfer method described here could eliminate the problems associated with vector-mediated gene transfer and allow functional expression of B7-1 or other molecules for use in many experimental and therapeutic applications. Other desirable features of this protein transfer method are that live cells are not needed and incorporation can be completed within a short time, if the appropriate GPI-anchored molecules are available. The level of expression can also be easily controlled by varying the concentration of protein used, the temperature, and the time during protein transfer (30). Cell membrane preparations, RBCs, liposomes, or any amphiphilic/hydrophobic surface can be modified with lipid-anchored proteins by protein transfer. Therefore, isolated cell membranes, irradiated cells, or liposomes entrapped with proteins can be modified to express the appropriate GPI-anchored molecules for targeted delivery to antigen-presenting cells.

Many investigators are beginning to exploit the properties of GPI anchors by designing proteins to have these unique lipid tails (31, 32). MHC class I has been modified to be cell surface-anchored via GPI. Using *in vitro* assays, it has been shown that cells modified to express GPI-anchored MHC class I molecule-hepatitis B viral peptide complexes by protein transfer served as targets for CTLs (33). In this report, we have shown that GPI-B7-1-incorporated tumor cell membranes can induce tumor-specific T-cell responses and provide protective immunity from tumor challenge *in vivo*. To our knowledge, this report is the first demonstration of the use of protein transfer of GPI-anchored proteins to express new proteins on isolated cell membranes and induce immunity against a disease.

In addition to the incorporation of proteins that stimulate immune activity, proteins that down-regulate or modulate effector functions

⁴ Y.-C. Wang and P. Selvaraj, unpublished observations.

can be expressed on the cell surface by protein transfer. For example, using protein transfer, one could introduce an antigenic peptide presented by GPI-anchored MHC complexes on cells such as RBCs or liposomes, which lack costimulatory molecules, and use these vehicles to induce anergy in antigen-specific T lymphocytes. This may be beneficial for the treatment of autoimmune diseases or transplantation. Further exploitation and study of this method could enable researchers to create the optimal immunotherapeutic or immunomodulatory cell that can induce regression of established tumors, treat autoimmune disease, and potentially aid in the acceptance of tissue transplants.

ACKNOWLEDGMENTS

We thank Drs. Aftab Ansari, Robert Donahoe, Peter Jensen, and Aron Lukacher for critical review of the manuscript; Dr. Judy Kapp for providing EG7 tumor cells; and Mr. Nawaz Ahmed for valuable technical assistance. We dedicate this study to the memory of Dr. Kenneth W. Sell, whose support and enthusiasm for this work will be forever valued.

REFERENCES

- Mueller, D. L., Jenkins, M. K., and Schwartz, R. H. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annu. Rev. Immunol.*, **7**: 445–480, 1989.
- Ledbetter, J. A., Imboden, J. B., Schieven, G. L., Grosmaire, L. S., Rabinovitch, P. S., Lindsten, T., Thompson, C. B., and June, C. H. CD28 ligation in T-cell activation: evidence for two signal transduction pathways. *Blood*, **75**: 1531–1539, 1990.
- Linsley, P. S., Brady, W., Grosmaire, L., Aruffo, A., Damle, N. K., and Ledbetter, J. A. Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *J. Exp. Med.*, **173**: 721–730, 1991.
- Azuma, M., Cayabyab, M., Buck, D., Phillips, J. H., and Lanier, L. L. CD28 interaction with B7 costimulates primary allogeneic proliferative responses and cytotoxicity mediated by small, resting T lymphocytes. *J. Exp. Med.*, **175**: 353–360, 1992.
- Tan, P., Anasetti, C., Hansen, J. A., Melrose, J., Brunvand, M., Bradshaw, J., Ledbetter, J. A., and Linsley, P. S. Induction of alloantigen-specific hyporesponsiveness in human T lymphocytes by blocking interaction of CD28 with its natural ligand B7/BB1. *J. Exp. Med.*, **177**: 165–173, 1993.
- Gimmi, C. D., Freeman, G. J., Gribben, J. G., Gray, G., and Nadler, L. M. Human T-cell clonal anergy is induced by antigen presentation in the absence of B7 costimulation. *Proc. Natl. Acad. Sci. USA*, **90**: 6586–6590, 1993.
- Chen, L., Ashe, S., Brady, W. A., Hellstrom, I., Hellstrom, K. E., Ledbetter, J. A., McGowan, P., and Linsley, P. S. Costimulation of antitumor immunity by the B7 counter receptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell*, **71**: 1093–1102, 1992.
- Townsend, S. E., and Allison, J. P. Tumor rejection after direct costimulation of CD8+ T cells by B7-transfected melanoma cells. *Science (Washington DC)*, **259**: 368–370, 1993.
- Baskar, S., Ostrand Rosenberg, S., Nabavi, N., Nadler, L. M., Freeman, G. J., and Glimcher, L. H. Constitutive expression of B7 restores immunogenicity of tumor cells expressing truncated major histocompatibility complex class II molecules. *Proc. Natl. Acad. Sci. USA*, **90**: 5687–5690, 1993.
- Guo, Y., Wu, M., Chen, H., Wang, X., Liu, G., Li, G., Ma, J., and Sy, M. Effective tumor vaccine generated by fusion of hepatoma cells with activated B cells. *Science (Washington DC)*, **263**: 518–520, 1994.
- Chen, P. W., Shreedhar, V. K., and Ananthaswamy, H. N. Rejection of murine melanoma cells transfected with the intercellular adhesion molecule-1 gene. *Int. J. Oncol.*, **6**: 675–680, 1995.
- McHugh, R. S., Ahmed, S. N., Wang, Y.-C., Sell, K. W., and Selvaraj, P. Construction, purification and functional reconstitution on tumor cells of glycolipid-anchored human B7-1 (CD80). *Proc. Natl. Acad. Sci. USA*, **92**: 8059–8063, 1995.
- Brunschwig, E. B., Levine, E., Trefzer, U., and Tykocinski, M. L. Glycosylphosphatidylinositol-modified murine B7-1 and B7-2 retain costimulator function. *J. Immunol.*, **155**: 5498–5505, 1995.
- Medof, M. E., Kinoshita, T., and Nussenzweig, V. Inhibition of complement activation on the surface of cells after incorporation of decay-accelerating factor (DAF) into their membranes. *J. Exp. Med.*, **160**: 1558–1563, 1984.
- Selvaraj, P., Dustin, M. L., Silber, R., Low, M. G., and Springer, T. A. Deficiency of lymphocyte function associated antigen-3 (LFA-3) in paroxysmal nocturnal hemoglobinuria: functional correlates and evidence for a phosphatidylinositol membrane anchor. *J. Exp. Med.*, **166**: 1011–1025, 1987.
- Moore, M. W., Carbone, F. R., and Bevan, M. J. Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell*, **54**: 777–785, 1988.
- Zhou, F., Rouse, B. T., and Huang, L. Prolonged survival of thymoma-bearing mice after vaccination with a soluble protein antigen entrapped in liposomes: a model study. *Cancer Res.*, **52**: 6287–6291, 1992.
- Maeda, T., Balakrishnan, K., and Mehdi, S. Q. A simple and rapid method for the preparation of plasma membranes. *Biochim. Biophys. Acta*, **731**: 115–120, 1983.
- Pardoll, D. M. Paracrine cytokine adjuvants in cancer immunotherapy. *Annu. Rev. Immunol.*, **13**: 399–415, 1995.
- Trinchieri, G. Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. *Blood*, **84**: 4008–4027, 1994.
- Coughlin, C. M., Wysocka, M., Kurzawa, H. L., Lee, W. M., Trinchieri, G., and Eck, S. L. B7-1 and interleukin 12 synergistically induce effective antitumor immunity. *Cancer Res.*, **55**: 4980–4987, 1995.
- Selvaraj, P., Rosse, W. F., Silber, R., and Springer, T. A. The major Fc receptor in blood has a phosphatidylinositol anchor and is deficient in paroxysmal nocturnal hemoglobinuria. *Nature (Lond.)*, **333**: 565–567, 1988.
- Chen, L., McGowan, P., Ashe, S., Johnston, J., Hellstrom, I., and Hellstrom, K. B7-1/CD80-transduced tumor cells elicit better systemic immunity than wild-type tumor cells admixed with *Corynebacterium parvum*. *Cancer Res.*, **54**: 5420–5423, 1994.
- Townsend, S. E., Su, F. W., Atherton, J. M., and Allison, J. P. Specificity and longevity of antitumor immune responses induced by B7-transfected tumors. *Cancer Res.*, **54**: 6477–6483, 1994.
- Brian, A. A. Stimulation of B-cell proliferation by membrane-associated molecules from activated T cells. *Proc. Natl. Acad. Sci. USA*, **85**: 564–568, 1988.
- Hodgkin, P. D., Yamashita, L. C., Seymour, B., Coffman, R. L., and Kehry, M. R. Membranes from both Th1 and Th2 T cell clones stimulate B cell proliferation and prepare B cells for lymphokine-induced differentiation to secrete Ig. *J. Immunol.*, **147**: 3696–3702, 1991.
- Ada, G. L. Vaccines. In: W. E. Paul (ed.), *Fundamental Immunology*, pp. 1309–1352. New York: Raven Press, Ltd., 1993.
- Nabel, E. G., Plautz, G., and Nabel, G. J. Transduction of a foreign histocompatibility gene into the arterial wall induces vasculitis. *Med. Sci.*, **89**: 5157–5161, 1992.
- Davis, H. L., Demeneix, B. A., Quantin, B., Coulombe, J., and Whalen, R. G. Plasmid DNA is superior to viral vectors for direct gene transfer into adult mouse skeletal muscle. *Hum. Gene Ther.*, **4**: 733–740, 1993.
- Nagarajan, S., Anderson, M., Ahmed, S. N., Sell, K. W., and Selvaraj, P. Purification and optimization of functional reconstitution on the surface of leukemic cell lines of GPI-anchored Fcγ receptor III. *J. Immunol. Methods*, **184**: 241–251, 1995.
- Ilangkumaran, S., Robinson, P. J., and Hoessli, D. C. Transfer of exogenous glycosylphosphatidylinositol (GPI)-linked molecules to plasma membranes. *Trends. Cell Biol.*, **6**: 163–167, 1996.
- Medof, M. E., Nagarajan, S., and Tykocinski, M. L. Cell-surface engineering with GPI-anchored proteins. *FASEB J.*, **10**: 574–586, 1996.
- Huang, J.-H., Getty, R. R., Chisari, F. V., Fowler, P., Greenspan, N. S., and Tykocinski, M. L. Protein transfer of preformed MHC-peptide complexes sensitizes target cells to T cell cytotoxicity. *Immunity*, **1**: 607–613, 1994.

CD8⁺ T Cells Activated During the Course of Murine Acute Myelogenous Leukemia Elicit Therapeutic Responses to Late B7 Vaccines After Cytoreductive Treatment

By Kyriaki Dunussi-Joannopoulos, Werner Krenger, Howard J. Weinstein, James L.M. Ferrara, and James M. Croop

We have previously shown in a murine acute myelogenous leukemia (AML) model that leukemic mice can be cured with a B7 vaccine if immunized early in the disease and that CD8⁺ T cells are necessary for tumor rejection. However, when B7 vaccine is administered 2 weeks after leukemia inoculation, the effect is only prolonged survival, ending in death virtually of all the mice. To distinguish between tumor kinetics and tumor-induced immunosuppression as potential mechanisms eliminating the therapeutic potential of late B7 vaccines, we performed *in vitro* T-cell studies during leukemia progression and *in vivo* studies on the clinical outcome of late B7 vaccines in combination with prior cytoreductive chemotherapy. Our results show that CD8⁺ T cells from leukemic mice 1 and 2 weeks after leukemia inoculation proliferate more vigorously in response to *in vitro* activation than

cells from normal mice and produce Th1-type cytokines interleukin-2 and interferon- γ . Cytotoxic T lymphocyte (CTL) assays demonstrate that cells from week-2 vaccinated mice (which succumb to their leukemia), surprisingly develop a stronger CTL activity than cells from week-1 vaccinated mice (which reject their leukemia). Finally, the combination of late chemotherapy and late B7 vaccine administration can cure only 20% of leukemic mice, whereas early chemotherapy and the same late B7 vaccine administration cure 100% of leukemic mice. These results demonstrate that in murine AML tumor growth does not induce T-cell anergy or a Th2 cytokine profile and suggest that tumor growth is most likely to be the limiting factor in the curative potential of late B7 vaccines.

© 1997 by The American Society of Hematology.

FOR SEVERAL DECADES it was believed that the failure of the immune system to exert an effective surveillance in cancer patients and tumor-bearing animals arises from the lack of tumor-specific antigen expression on tumor cells. Accumulating evidence¹⁻⁸ now suggests that this failure does not result from the absence of recognizable tumor antigens but, rather, from the inability of tumor cells to either initiate or complete an effective immune cytolytic response. Primary factors that have been implicated for this unfavorable host-tumor relationship are the inability of most of the tumor cells to provide T-cell costimulatory signals,^{9,10} and the absence of an appropriate cytokine microenvironment.¹¹⁻¹³

A successful antitumor T-cell response involves the induction, recruitment, and effector functions of tumor-specific T cells and requires at least two distinct signals: a signal through the T-cell receptor (TcR) complex, and a costimulatory signal provided by the ligation of adhesion molecules on T cells with their counterreceptors on the surface of professional antigen-presenting cells (APCs).^{14,15} The CD28 signaling pathway is the most potent costimulatory pathway identified to date and is activated by binding of CD28 on T cells to either B7.1 (CD80) or B7.2 (CD86) expressed on the surface of professional APCs.^{16,17} Several studies in murine tumor models have shown that ectopic expression of the members of the B7 family of T-cell costimulatory molecules by tumor cells is effective at inducing protective immunity against a number of murine tumors.¹⁸⁻²² Recently, we have shown in a murine acute myelogenous leukemia (AML) model, that one intravenous (IV) injection of irradiated B7.1⁺ AML cells can provide mice with long-lasting systemic immunity against subsequent challenge with wild-type AML cells, and that CD8⁺ T cells are necessary for the tumor-specific immune response.²³ Furthermore, one exposure to irradiated, B7.1⁺ AML cells can cure leukemic mice vaccinated early (1 week) after leukemia inoculation, whereas late (2 week) vaccinations only delay tumor growth. This discrepancy is consistent with other model systems, where an effective immune response could only be achieved against small pre-existing tumor burdens.^{18,21,22} These results

have provoked controversy, as to whether tumor growth induces mechanisms of peripheral tolerance to cancer, thus eliminating the therapeutic potential of late vaccines. In support of a dysregulated immune system are reports of cancer patients with progressive disease and marked decreases in T-cell responses,^{24,25} and of T cells from both patients and tumor-bearing mice that produce Th2 (IL-4, IL-10) cytokines,^{26,27} which can inhibit a cell-mediated antitumor immune response.²⁸

Although it is not yet known how T cells "interact" with tumor antigens *in vivo*, it has been speculated but not demonstrated that these interactions can induce tolerance in the host.²⁹ Moreover, there is a scant information pertaining to the functional properties of T cells in response to hematopoietic tumor models. In murine AML tumor models, AML cells home to bone marrow and spleen after IV inoculation. This very close interaction between AML cells and T cells from the initial stages of the disease, in a presumably different cytokine and APC microenvironment than in any solid tumor model, could affect the function of the T cells in an unpredictable manner.

The current studies were undertaken to distinguish be-

From the Division of Pediatric Oncology, Dana-Farber Cancer Institute, Department of Pediatrics, and the Division of Pediatric Oncology, Department of Pediatrics, Massachusetts General Hospital, Harvard Medical School, Boston, MA.

Submitted September 23, 1996; accepted November 14, 1996.

Supported in part by the Andrew F. Gaffney Foundation, the Claudia Adams Barr Program of the Dana-Farber Cancer Institute. JLMF is a scholar of the Leukemia Society of America.

Address reprint requests to Kyriaki Dunussi-Joannopoulos, MD, PhD, Division of Pediatric Oncology, Dana-Farber Cancer Institute, 44 Binney St, Boston, MA, 02115.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1997 by The American Society of Hematology.
0006-4971/97/8908-0012\$3.00/0

tween rapid tumor growth and immunosuppression as potential mechanisms eliminating the therapeutic potential of late B7 vaccines and to investigate if the combination of cytoreductive chemotherapy and B7 vaccines can cure mice with advanced AML. We studied the *in vitro* functional properties of splenic CD8⁺ cells from mice with different tumor burdens (week 1 or 2 after AML inoculation) and the CTL activity of CD8⁺ T cells from mice vaccinated at week 1 or 2 of the AML course, previously shown to have different clinical outcomes. We show that CD8⁺ T cells from leukemic mice respond more vigorously than cells from normal mice to *in vitro* activation and produce Th1-type cytokines. The cytotoxic T lymphocyte (CTL) assays demonstrate that, despite the nontherapeutic clinical outcome of mice vaccinated at week 2, cells from these mice generate a stronger cytolytic response than cells from mice vaccinated at week 1, suggesting that progressive growth of tumor induces mechanisms that defeat a tumor-specific generated CTL activity. Finally, we show that the combination of chemotherapy, to reduce tumor burden, and B7 vaccines can lead to more effective clinical responses of mice with advanced disease.

MATERIALS AND METHODS

Mice. Female SJL/J mice, 6 to 8 weeks old, were purchased from Jackson Laboratories (Bar Harbor, ME) or Charles River Laboratories (NCI-Frederick Cancer Research & Development Center Frederick, MD). The animals were kept at the animal facility of Dana-Farber Cancer Institute (Boston, MA) according to the Institute's guidelines.

Murine AML model. The murine AML model used in this study has been previously described.²³ Briefly, AML cells, originally obtained from radiation-induced AML in female SJL/J mice,³⁰ are maintained by growing in syngeneic SJL/J mice. Mice injected IV or intraperitoneally (IP) with $\geq 10^4$ AML develop leukemia and die in 4 to 5 weeks. In all experiments, freshly isolated or frozen spleen mononuclear cells from leukemic mice (4 to 5 weeks after the tumor inoculation) were used. Flow cytometry results demonstrated that these cells were essentially 100% myeloblasts.²³

Purification of CD8⁺ T cells. Spleens from naive or leukemic SJL/J mice were removed aseptically and single cell suspensions were obtained on ice by gently pressing tissue pieces against the bottom of a petri dish with a syringe plunger. Cells were stained with Ly-2 monoclonal antibody (MoAb) (PharMingen, San Diego, CA), labeled with goat antirat IgG Microbeads (Milteny; Biotec, Sunnyvale, CA) and selected using magnetic MiniMacs separation columns (Milteny). Isolated cells were 90% to 94% pure as determined by immunofluorescent flow cytometry analysis (FACS) and appeared viable by exclusion of trypan blue and forward/side scatter analysis. The absolute numbers of purified CD8⁺ T cells from week 1 and week 2 leukemic mice were approximately the same.

Proliferation assays and reagents. Purified CD8⁺ T cells were cultured at 2×10^5 cells/well in U-bottomed 96-well plates in the presence of Concanavalin A (ConA; 2.5 μ g/mL) or phorbol 12-myristate 13-acetate (PMA; 10 ng/mL) plus ionomycin (300 ng/mL). For TcR-crosslinking of CD8⁺ responder cells, wells were precoated with anti-CD3 MoAb 145-2C11 (2.5 μ g/mL) for 90 minutes at 37°C. A feeder layer of 2×10^5 syngeneic, anti-Thy 1.2 and C'-treated and irradiated (2,000 rad) splenocytes was also added to each well. Supernatants were collected after 24 and 48 hours of culture and were assayed for cytokine levels as described below. Proliferation

of responder cells was measured after 72 hours by the incorporation of ³H thymidine (1 μ Ci/well) for the last 20 hours of incubation.

ConA, PMA, and ionomycin were purchased from Sigma Chemical Co (St Louis, MO). The anti-Thy 1.2 MoAb used for T-cell depletion was isolated from tissue culture supernatants from hybridoma clone HO-13-4 (American Type Culture Collection, Rockville, MD; TIB # 99).³¹ Low-Tox-M rabbit complement (C') was obtained from Accurate Corp (Westbury, NY). The anti-CD3 MoAb (145-2C11) was a generous gift from Dr S.J. Burakoff (Dana-Farber Cancer Institute).

Lymphokine ELISAs. Levels of interleukin-2 (IL-2), interferon- γ (IFN- γ), and IL-4 in tissue culture supernatants were determined by sandwich ELISA using specific antimurine MoAbs for capture and detection (PharMingen). A color reaction was developed using streptavidin-conjugated horseradish peroxidase (Genzyme, Cambridge, MA) followed by TMB peroxidase substrate (Kirkegaard & Perry Laboratories, Inc, Gaithersburg, MD). A standard curve was generated to determine the cytokine concentration in the sample. The lower detection limit of all cytokine assays was 1.0 U/mL. Each assay was performed in triplicate at least three times, and the results from a typical assay are reported.

The following MoAbs for capture and detection were used: IL-2, purified JES6-1A12 and biotin-conjugated JES6-5H4³²; IFN- γ , purified R4-6A2³³ and biotin-conjugated XMG1.2³⁴; IL-4, purified 11B11³⁵ and biotin-conjugated BVD6-24G2.³² Recombinant murine IL-2 (rIL-2) was obtained from Boehringer Mannheim (Indianapolis, IN). Murine rIFN- γ was purchased from Amgen (Thousand Oaks, CA) and murine rIL-4 with a specific activity of 7×10^7 U/ μ g by FDCP-2 assay was obtained from Immunex Corp (Seattle, WA).

⁵¹Cr release assays. One or 2 weeks following injection of AML cells the mice were immunized with irradiated 10^5 B7-AML cells. One week after immunization their splenocytes were harvested and nylon-wool-enriched T cells were depleted of CD4⁺ and GR-1⁺ cells by magnetic cell separation. The resulting population of cells was used as effectors for the CTL assays. We could not detect any differences in the absolute numbers of purified CD8⁺ T cells from week 1 and week 2 vaccinated mice. FACS analysis showed that these cells were 90% to 94% CD8⁺ cells. Autologous AML cells or control EL-4 tumor cells were incubated with ⁵¹Cr (New England Nuclear, Boston, MA) for 2 hours and used as targets in the CTL assays.

The standard 4-hour CTL assays were set up with various effector to target (E:T) ratios in a total volume of 0.2 mL/well in a 96-well microtiter plate. All conditions were set up in quadruplicate. After a 4-hour incubation, 100 μ L of supernatant was harvested from each well, and the quantity of ⁵¹Cr in the supernatants was determined using a gamma counter. Results are expressed as the percentage of specific lysis, calculated as $100 \times (\text{sample cpm} - \text{spontaneous cpm}) / (\text{maximum cpm} - \text{spontaneous cpm})$. The cpm of supernatant from wells containing target cells in normal media and from wells containing target cells in 1% Triton X-100 served as the spontaneous and the maximum release, respectively.

In vivo immunization studies. SJL/J mice were injected IV with live or irradiated (3,200 rad from a ¹³⁷Cs source) B7.1 transduced AML cells. Expression of B7.1 on AML cells has been previously described.²³ Briefly, E-86 cells were transduced with the LNCX-B7.1-sense or LNCX-B7.1-antisense (mock virus) retroviral constructs and two clones (B7.1 sense and B7.1 antisense), secreting high titers of virus, were used to infect the AML cells. AML cells (3 to 5×10^5 cells/mL) were exposed to viral supernatant for 24 to 48 hours. Infected, unselected AML cells were washed in phosphate buffered saline (PBS), counted, and the designated numbers of cells were used for the *in vivo* immunizations.

Cytoreductive treatment. Mice were injected IV in the tail vein

with 10^5 wild-type AML cells in 0.3 ml saline. On days 7, 10, 14 (protocol I) or 3, 6, and 8 (protocol II) post AML inoculation, they received two consecutive IP injections of 200 mg/kg Ara-C (CHIRON Therapeutics, Emeryville, CA), 6 hours apart. Treated mice were vaccinated on day 16 after leukemia inoculation with B7-AML cells. Some mice remained without vaccination and 1 or 2 weeks after the end of chemotherapy (corresponding to 2 or 3 weeks post AML inoculation) they were killed and their spleen and bone marrow cells were stained with the GR-1 MoAb and analyzed by immunofluorescent flow cytometry.

Immunostaining and flow cytometry analysis. Cells were stained as previously described.²³ The following antibodies were used in this study: CD80 (B7.1), Gr-1, CD4 (L3T4), CD8a (Ly-2), (PharMingen). After staining, the cells were fixed in 2% paraformaldehyde and analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Statistical analysis. Values are mean \pm SEM. The statistical significance between any two groups was analyzed by Student's *t*-test.

RESULTS

CD8⁺ T cells from leukemic SJL/J mice proliferate more vigorously than CD8⁺ T cells from normal SJL/J mice. In vivo depletion studies have shown that, in this AML model, rejection of the leukemic cells by vaccinated mice requires the presence of CD8⁺ but not of CD4⁺ T cells.²³ Therefore, we studied the functional properties of CD8⁺ T cells from leukemic mice at 1 week (T-1) or 2 weeks (T-2) after leukemia inoculation and compared them with the functional properties of cells from normal mice (T-N). We examined the capacity of T-1, T-2, and T-N cells to proliferate in response to in vitro activation with: (1) a high density/high avidity TcR signal, provided by plate-bound anti-CD3; (2) ConA; and (3) PMA plus ionomycin. As shown in Fig 1A, T-1 cells showed 57% higher proliferative response than T-N in response to activation with anti-CD3 ($P < .01$), 39% higher response to ConA ($P < .01$), and 28% higher response to PMA plus ionomycin ($P < .01$). Similarly, T-2 cells had 55%, 41%, and 58% higher response than T-N cells in response to stimulation with anti-CD3, ConA, and PMA plus ionomycin, respectively (Fig 1B). These results revealed that T-1 and T-2 cells responded more vigorously than T-N cells not only to PMA plus ionomycin, a signal known to bypass the need for TcR stimulation by activating distal to PKC signaling pathways, but also to TcR stimulation (ConA or anti-CD3 MoAb), suggesting that the majority of T-1 and T-2 cells were not anergic.

CD8⁺ T cells from leukemic SJL/J mice have a Th1 cytokine profile. Protective immune responses are coordinated to a large extent by cytokines produced by Th1 and Th2 T-cell subsets,^{36,37} whereas the role of the recently recognized Th0 subset³⁸ has still to be defined. Th1 cells secreting IL-2 and IFN- γ induce cellular immune responses, whereas Th2 cells producing IL-4, IL-5, IL-6, and IL-10, promote humoral immune responses.^{39,40} It has been speculated that one possible mechanism leading to inability of an intact host to eliminate autologous tumor growth is the gradual loss of the Th1 cytokine profile and shift to Th2 type.^{26,27} We therefore examined the cytokine profile of splenic CD8⁺ T cells in our AML model and found that both T-1 and T-2 cells

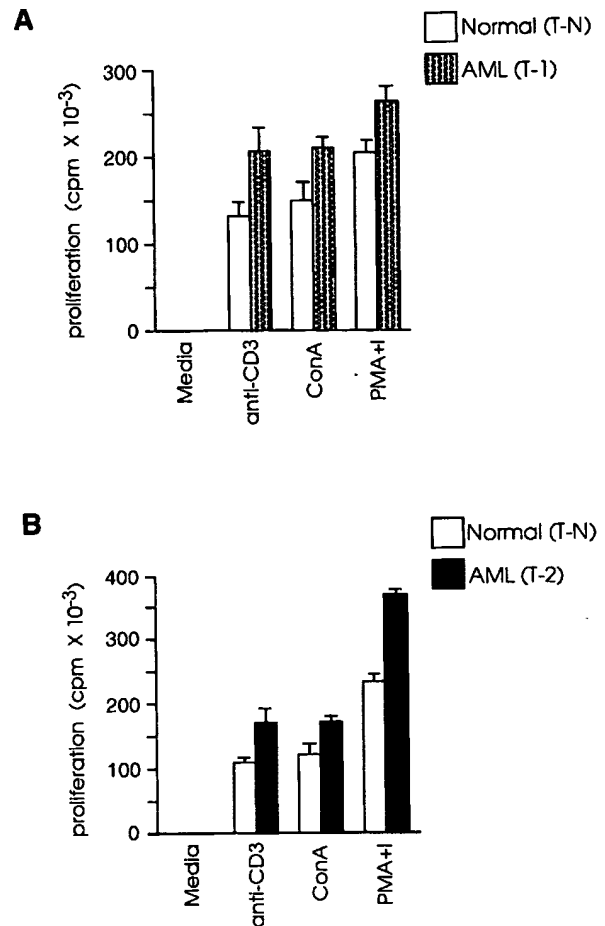


Fig 1. CD8⁺ T cells from leukemic mice proliferate more vigorously than CD8⁺ T cells from normal mice. Splenic CD8⁺ T cells were isolated from normal and leukemic SJL/J mice, 1 week (T-1) or 2 weeks (T-2) after leukemia injection, by magnetic cell separation. T-1 (A) and T-2 (B) cells were cultured at 2×10^5 cells/well in the presence of ConA (2.5 μ g/mL), PMA (10 ng/mL) plus ionomycin (300 ng/mL) or anti-CD3 MoAb (2.5 μ g/mL) and a feeder layer of 2×10^5 syngeneic, anti-Thy 1.2 and C'-treated and irradiated (2,000 rad) splenocytes. ³H thymidine (1 μ Ci/well) was added during the last 20 hours of culture. Results are representative of three independent experiments (each experiment included two to three mice) and are shown as the mean and SD of triplicate culture.

produced Th1 type cytokines, whereas the level of IL-4 production by CD8⁺ T cells from normal and leukemic mice was very low to undetectable (Fig 2A). Secretion of IFN- γ by T-1 cells was 82% higher than T-N cells ($P < .005$), and IL-2 levels produced by T-1 cells were 51% higher than those produced by T-N cells ($P < .01$). The differences in cytokine secretion were even more prominent when T-2 cells were used in the experiments. As shown in Fig 2B, IFN- γ levels produced by T-2 cells were nearly three times those produced by T-N cells ($P < .001$), and IL-2 production by T-2 cells was more than twice as great as that produced by T-N cells ($P < .0001$). We further examined the cytokine

profile of T-1 and T-2 cells in response to anti-CD3 and ConA stimulation. As shown in Fig 3A, CD8⁺ T cells from both normal and leukemic mice produced low levels of IL-2 in response to anti-CD3 and ConA, as opposed to stimulation with PMA plus ionomycin. Figure 3B shows that T-1 and T-2 cells produced equivalent or higher levels of IFN- γ than normal cells in response to anti-CD3 and ConA, but the most striking differences were observed when the cells were stimulated with PMA plus ionomycin.

CD8⁺ T cells from mice vaccinated at week 2 have higher CTL activity than cells from mice vaccinated at week 1. We examined the CTL activity of CD8⁺ T cells from mice vaccinated at week 1 or 2 after leukemia injection; the former group of mice shows a curative response, whereas the latter group has only prolonged survival.²³ As shown in Fig 4A, splenic CD8⁺ T cells isolated from mice a week after their

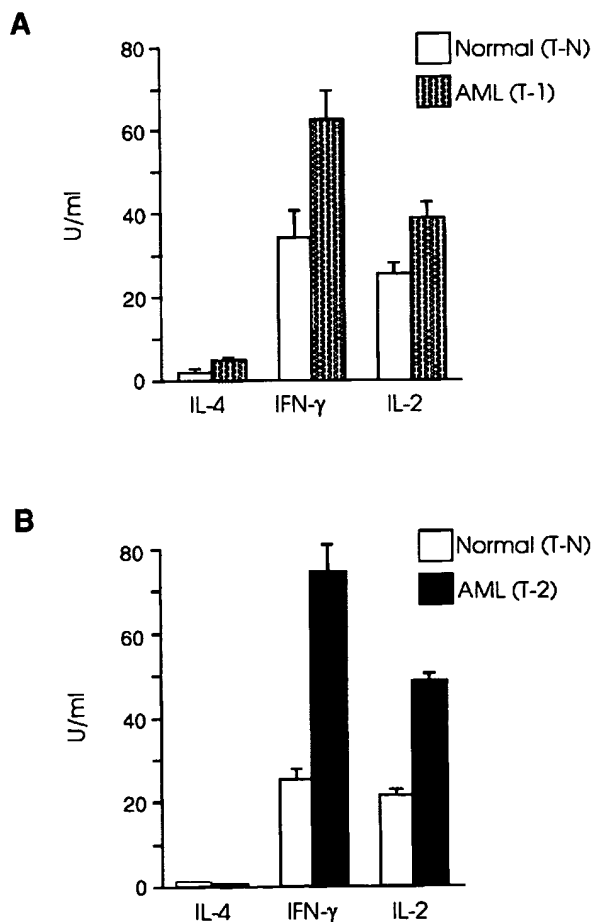


Fig 2. CD8⁺ T cells from leukemic SJL/J mice secrete Th1 type cytokines. (A) T-1 and (B) T-2 cells were cultured at 2×10^5 cells/well in the presence of PMA plus ionomycin and a feeder layer of irradiated 2×10^5 syngeneic, anti-Thy 1.2 and C'-treated splenocytes. Cytokine concentrations were determined in tissue culture supernatants taken after 24 hours (IL-2) or 48 hours (IFN- γ , IL-4) of the initiation of the culture. The graphs are representative of three independent experiments. Data are shown as the mean and SD of triplicate culture.

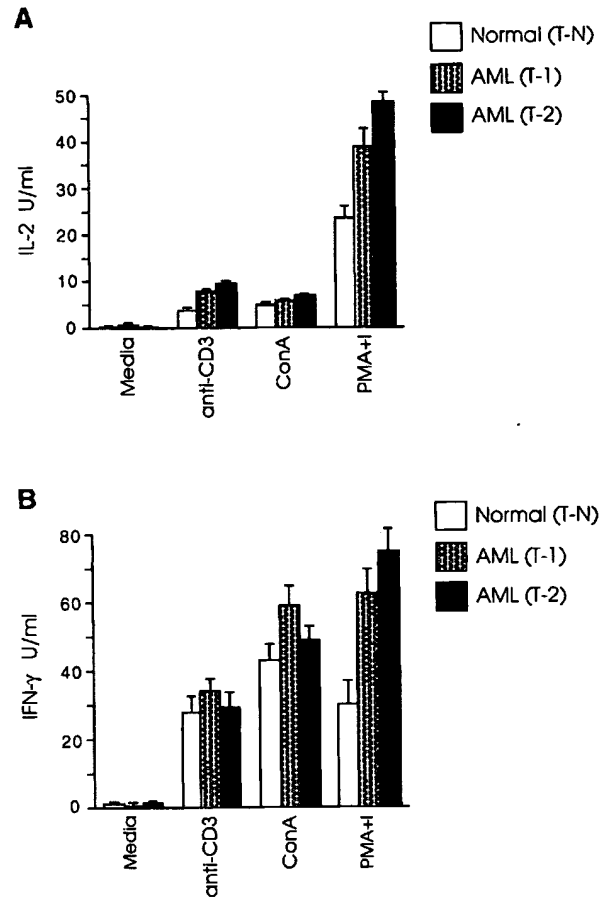


Fig 3. CD8⁺ T cells from leukemic mice secrete equal or higher levels of Th1 type cytokines than normal cells in response to TcR and mitogen stimulation. T-1 and T-2 cells were cultured at 2×10^5 cells/well in the presence of anti-CD3 or mitogens and a feeder layer of irradiated 2×10^5 syngeneic, anti-Thy 1.2 and C'-treated splenocytes. (A) IL-2 and (B) IFN- γ levels were determined as described in Materials and Methods. The graphs represent three separate experiments. Data are shown as the mean and SD of triplicate culture.

vaccination with B7-AML cells generated a strong cytolytic response on stimulation with wild-type AML cells in the presence of 5 U/mL exogenous IL-2 and in the absence of APCs (costimulatory signals). When under the same experimental conditions splenic CD8⁺ T cells from mice vaccinated at week 2 were studied, surprisingly these cells generated a stronger *in vitro* cytolytic response, which at a 100:1 ratio of E:T cells was 100% (Fig 4B). The response was AML-specific because the same cells did not lyse alloantigen presenting EL-4 (H-2^b) cells. These findings suggest that an anti-AML effector CTL population had been generated *in vivo* and that APC-derived costimulatory signals and restimulation were not required for the *in vitro* activation.

Cytoreductive treatment improves the vaccination potential of the leukemic cells. Tumor cell kinetics has been considered as a possible mechanism limiting the efficacy of tumor vaccines in animals with large tumor burdens.¹² Based

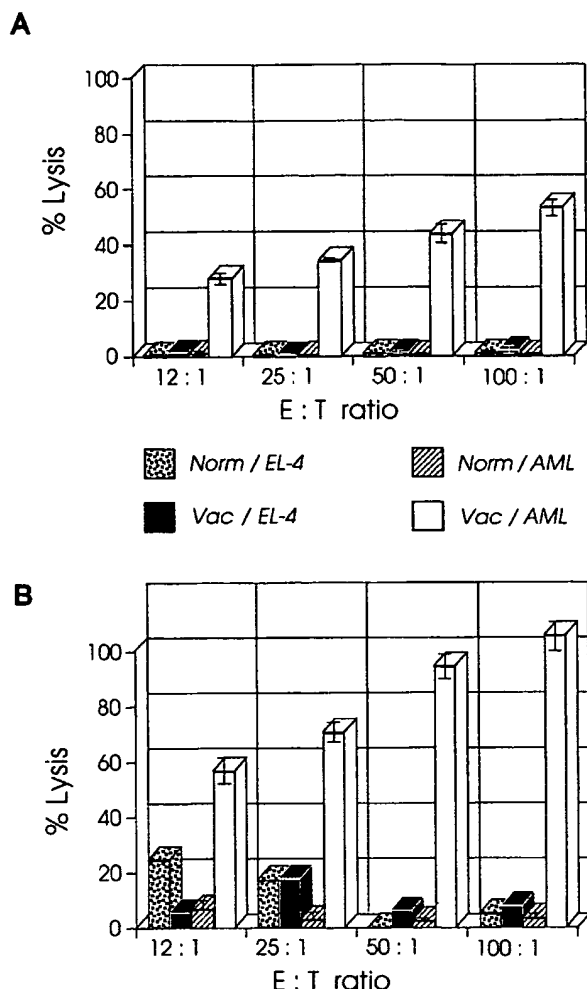


Fig 4. CTL activity of CD8⁺ T cells from week 2 vaccinated mice is higher than CTL activity from week 1 vaccinated mice. SJL/J mice were injected with 10⁵ AML cells. One or 2 weeks later they were immunized with irradiated 10⁵ B7-AML cells. One week after immunization CD8⁺ splenocytes were isolated as described in Materials and Methods and used as effector cells in various effector to target (E:T) ratios. Target cells (autologous AML or control EL-4 cells) were incubated with ⁵¹Cr for 2 hours. The standard 4-hour CTL assays were set up with various E:T ratios in a total volume of 0.2 mL/well in a 96-well microtiter plate. All conditions were set up in quadruplicate. After a 4-hour incubation, 100 μ L of supernatant was harvested from each well, and the quantity of ⁵¹Cr in the supernatants was determined. (A) CTL activity of CD8⁺ cells from week 1 vaccinated mice on autologous AML cells (Vac/AML) and control EL-4 cells (Vac/EL-4). In the same experiment, CD8⁺ cells from normal SJL/J mice were tested for CTL activity on autologous AML cells (Norm/AML) and control EL-4 cells (Norm/EL-4). (B) CTL activity of CD8⁺ cells from week 2 vaccinated mice on autologous AML cells (Vac/AML) and control EL-4 cells (Vac/EL-4). Control cells from normal SJL/J mice were used as described in graph A.

on our findings that vaccination of leukemic mice 2 weeks after leukemia inoculation had limited therapeutic effect but that the CTL response was still vigorous, we combined week 2 vaccines with prior cytoreductive treatment to determine

if a better therapeutic effect could be generated. We treated mice with Ara-C, 2 \times 200 mg/kg, administered 6 hours apart,⁴¹ on days 7, 10, and 14 (protocol I) or days 3, 6, and 8 (protocol II) after leukemia injection. These mice were then vaccinated with 10⁵ irradiated B7-AML cells on day 16 after leukemia injection. As controls we used two groups of leukemic mice: (1) mice that received no treatment, and (2) mice that received chemotherapy but no vaccine. In order to confirm that our chemotherapy protocol was reducing the tumor burden, we treated groups of leukemic mice with either protocol I or II, and 1 or 2 weeks after the end of chemotherapy we analyzed their bone marrow and spleen cells using the GR-1 myeloid differentiation antigen as a phenotypic marker (not found on lymphoid or erythroid cells), as previously described.²³ Mice treated with protocol II (days 3, 6, and 8) had almost no GR-1 positive bone marrow and spleen cells 1 week after the end of chemotherapy (0.8%) and had a low number of GR-1 positive cells (11%) 2 weeks later. The vast majority of bone marrow cells were CD3⁺ T cells (data not shown). However, mice treated with protocol I (days 7, 10, and 14) had a very small number of GR-1 positive cells in their bone marrow and spleen a week after the end of chemotherapy, but 2 weeks after chemotherapy both bone marrow and spleen were heavily infiltrated by leukemic cells (Fig 5A and B).

We have been able to improve the efficacy of late B7 vaccines by combining their administration with cytoreductive treatment. Two chemotherapy protocols were used and survival of chemotherapy-treated and vaccinated mice is illustrated in Fig 6. In the protocol I experiment, 20% of chemotherapy-treated and vaccinated mice rejected their tumor completely and 80% had 2 to 3 weeks prolonged survival compared with control leukemic mice, which received chemotherapy but no vaccine. The chemotherapy-treated but not vaccinated control mice had only 1 week prolonged survival compared with nontreated leukemic mice (Fig 6A). On the contrary, when protocol II was used for treatment, the combination of chemotherapy and B7 vaccine led to the cure of 100% of the leukemic mice. As shown in Fig 6B, mice treated only with chemotherapy had a 3-week prolonged survival compared with nontreated leukemic mice, whereas 100% of the mice that received both chemotherapy and B7 vaccine rejected their leukemia.

DISCUSSION

The experiments described in this report were motivated by recent studies on solid tumor models and our own study on a murine AML model demonstrating that B7 vaccines have curative potential only when administered to animals with small tumor burden. One speculation has been that tumor growth induces immunosuppressive mechanisms, with T-cell anergy or Th2 type cytokine profile of effector cells as potential candidates. Here we demonstrate that, in this AML model, tumor inoculation into naive mice induces activation rather than tolerance of CD8⁺ spleen cells during the first half of the leukemic course, and that the cells have a Th1 type cytokine profile. We also demonstrate that the limited clinical response of leukemic mice with large tumor burden

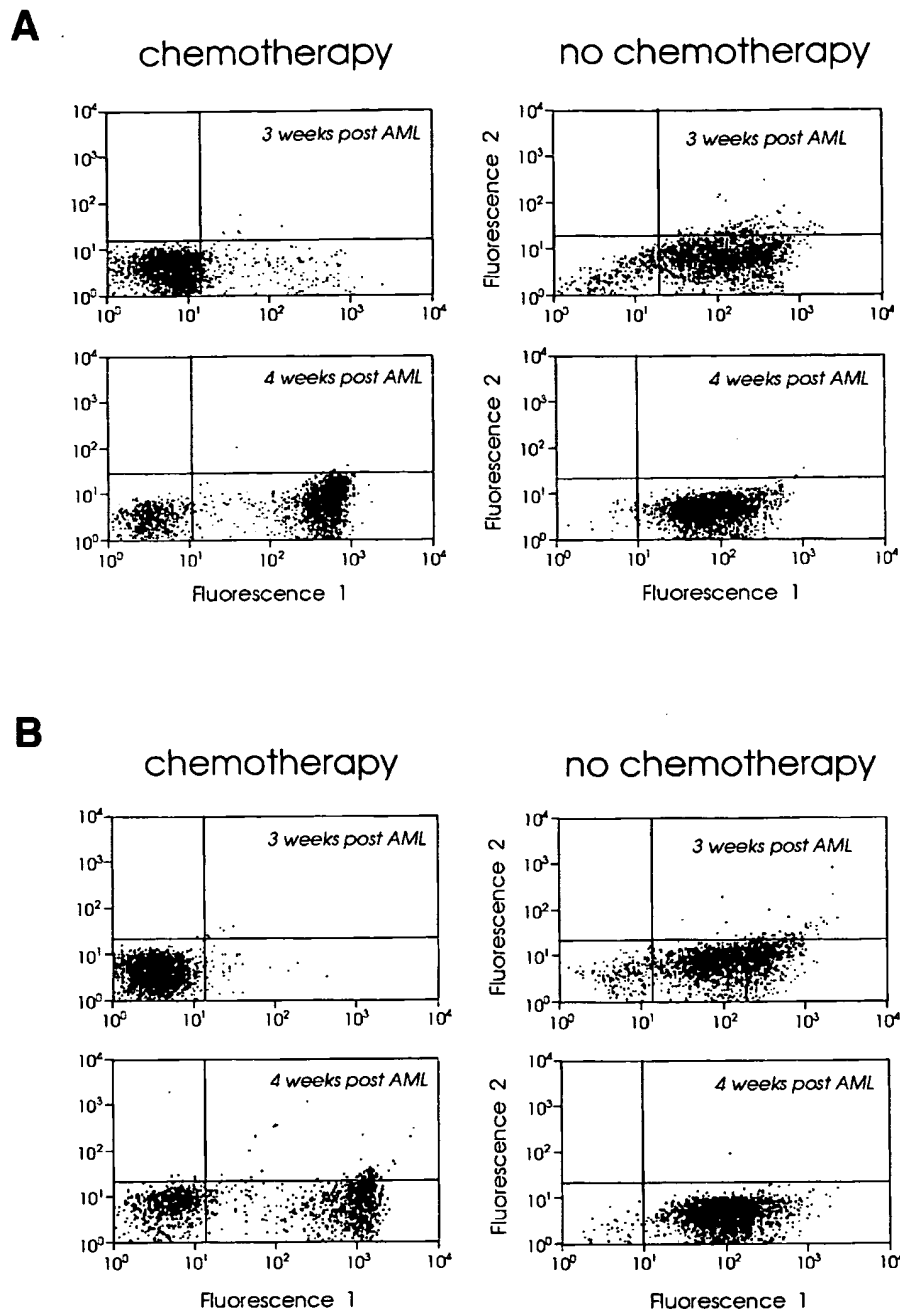


Fig 5. FACS analysis of (A) bone marrow and (B) spleen cells from leukemic SJL/J mice with chemotherapy (left panels) or without chemotherapy (right panels). SJL/J mice were injected with 10^5 AML cells and treated with chemotherapy on days 7, 10, and 14 (protocol I). One or 2 weeks after chemotherapy (3 or 4 weeks after leukemia inoculation), bone marrow and spleen cells were stained with Gr-1 MoAb or control Ig. A total of 5,000 cells were analyzed by FACS for each sample.

to B7 vaccines cannot be attributed to the lack of tumor-specific CTLs. Additional evidence that tumor kinetics overwhelm an ongoing immune response in this AML model is provided from chemotherapy treatment experiments. These experiments clearly demonstrate that reduction of the tumor burden during the first week after leukemia injection can cure 100% of the mice vaccinated at week 2. However, when chemotherapy is provided during the second week of the leukemic course, 80% of the vaccinated mice have prolonged survival while 20% reject their tumor.

It has been well documented in experimental model systems, that T cells become anergized when they are antigenically stimulated in the absence of costimulatory signals.^{42,43} Anergized T cells become incapable of proliferating and producing IL-2 on restimulation through their TcR and costimulatory ligands due to a defect in the TcR ζ -chain phosphorylation and its sequential interaction with ZAP-70 (both essential for the initiation of TcR-mediated signal transduction).^{44,45} This defect can be bypassed by stimulation of T cells with PMA plus ionomycin which activates distal

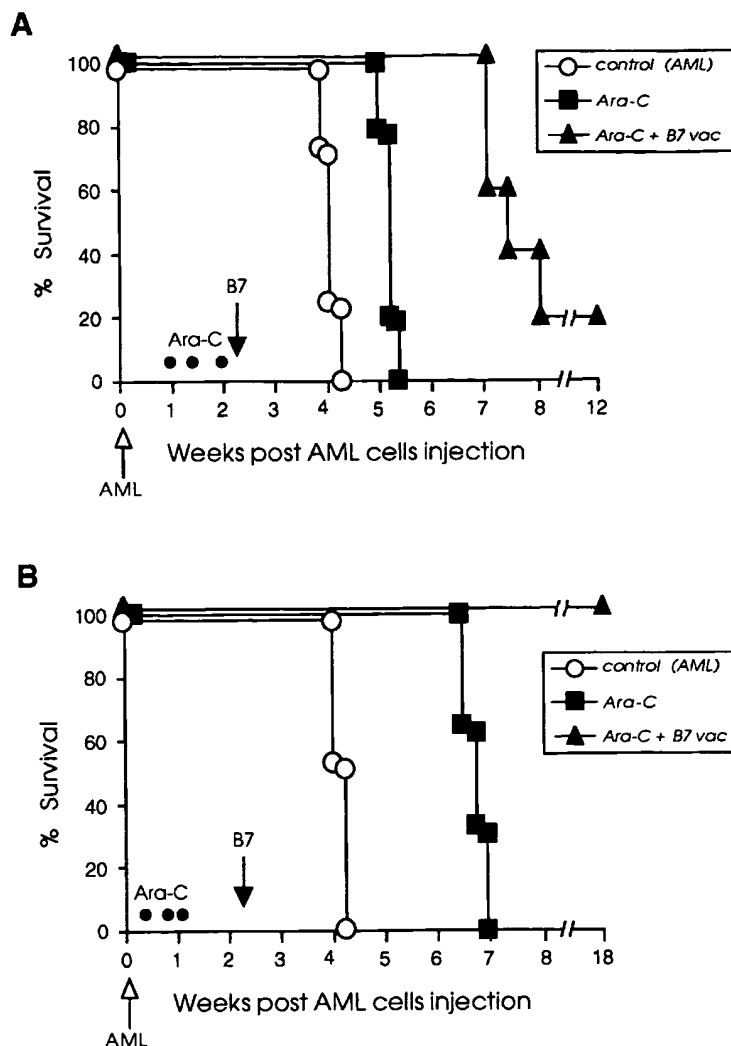


Fig 6. Cytoreductive treatment improves the vaccination potential of B7-AML cells. SJL/J mice were injected intravenously with 10^5 wild-type AML cells (open arrow) and then divided into three different groups. One group (○) received no further treatment. A second group (■) was treated with Ara-C (●) on days 7, 10, and 14 (protocol I) or 3, 6, and 8 (protocol II), as described in Materials and Methods, but was not vaccinated. A third group of leukemic mice (▲) was treated with Ara-C, and on day 16 (black arrow) was vaccinated with 10^5 irradiated B7-AML cells. Graph A illustrates the survival of mice treated with protocol I and graph B illustrates the survival of mice treated with protocol II. Each experimental and control group included 8 to 10 mice.

to PKC signaling pathways.⁴⁶ In our experiments, splenic CD8⁺ T cells from leukemic mice proliferated more vigorously than normal CD8⁺ T cells not only in response to activation with PMA plus ionomycin, but more important, to antigenic stimulation through TcR. Although these findings cannot exclude the possibility that some T cells were anergic, they demonstrate that on a population basis, a considerable percentage of the cells had encountered an antigenic stimulation in vivo and had thus entered an activated state.

Wild-type AML cells in this model do not express B7.1 or B7.2 costimulatory molecules and therefore they cannot directly activate T cells as professional APCs. Our observation that CD8⁺ T cells from leukemic mice were in an activated state raises a multitude of questions regarding tumor antigen presentation during the AML course: (1) are tumor antigens exclusively presented by host bone marrow-derived APCs, which are able to provide antigen and costimulatory signals to T cells? (2) Do T cells "see" tumor antigens on the AML cells, even in the absence of costimulatory signals,

thus becoming temporarily unresponsive due to TcR down-regulation? (3) Is the host immune response a continuous antagonism of the two aforementioned mechanisms, and could this explain why immunogenicity, route of injection, and number of injected cells play a specific role in the tumorigenicity of the tumor cells?

In support of the first hypothesis are two studies demonstrating that MHC class I-restricted tumor antigens are exclusively presented by host bone marrow-derived APCs⁴⁷ and that, even in B7.1 tumor vaccines, the dominant mechanism of CTL priming is through the uptake and presentation of tumor antigens by bone marrow-derived APCs.⁴⁸ If this is indeed the case, the number of surrounding APCs would have a very important impact on effectively taking up and presenting antigens provided by the growing tumor and would also explain why B7 vaccines can be therapeutic only under circumstances with relatively small tumor burden (especially in nonsolid tumors, in which the factor of a large, abnormally vascularized tumor mass, with limited traffic of

effector cells, is excluded). We are currently investigating in our model if the simultaneous administration of tumorigenic AML cells and bone marrow-derived dendritic cells⁴⁹ can prevent leukemia in naive hosts; and moreover, if the injection of autologous dendritic cells in leukemic mice can rescue them from lethal disease.

The second hypothesis, that T cells can recognize tumor-specific antigens, even in the absence of costimulatory molecules, is strengthened by recent observations that T-cell activation depends on the number of triggered TcRs, is independent of the nature of the triggering ligand, and that the activation threshold is decreased when costimulatory signals are provided.⁵⁰ In the reported experimental system, T cells expressing very few TcRs (resulting from TcR down-regulation due to antigenic stimulation and lasting 5 to 8 days⁵¹) are triggered transiently but fail to be activated, because the number of TcRs is insufficient to allow these cells to sustain the signal and reach the activation threshold. Our findings do not support this as the only mechanism of tumor antigen-T-cell interaction in the AML model. If T cells, being exposed to an increasing number of AML cells in the splenic microenvironment, can be directly stimulated by them in the absence of costimulatory signals, we would expect the majority of T cells to be unresponsive in our in vitro activation experiments and, furthermore, this number to increase with disease progress. Additional evidence that T-cell unresponsiveness is not induced by tumor growth in this AML model, and that tumor antigen presentation mediated by APCs plays a definite role (thus their number being critical for an effective immune response), is provided by our observations that: (1) $\geq 10^4$ is a tumorigenic dose when wild-type leukemic cells are injected IV or IP, but $> 10^5$ is the tumorigenic dose when the subcutaneous (SC) route is used for injection; (2) when mice are immunized with a nontumorigenic dose (10^4) of live cells SC, they are capable of rejecting a subsequent SC tumor challenge with 10^6 cells but not an IV or IP challenge with the same number of cells (Dunussi-Joannopoulos et al, unpublished data). This is in agreement with previous reports on immunogenic tumor models, in which mice immunized with the minimal tumorigenic dose of irradiated, B7-devoid tumor cells, do not get tolerized and are capable of rejecting a subsequent tumor challenge with live cells.^{52,53}

Another important issue is provided by the findings from the CTL assays. We have shown that the CTL activity of mice vaccinated at week 2 is surprisingly higher than the CTL activity of mice vaccinated at week 1 after leukemia injection and would expect the former group to reject leukemia as does the latter group. We can speculate that the effector CTL population in the vaccinated mice comprises a mixture of CTLs, part of which has been activated by APCs before vaccination and another part by the B7-AML cells provided by the vaccine. This can explain the presence of a higher number of effector T cells in the group of mice that have been vaccinated 2 weeks after leukemia injection and thus have had longer exposure to tumor antigen presenting APCs. Because mice vaccinated at week 2 finally fail to reject their leukemia despite the higher in vitro CTL activity,

we propose two potential mechanisms as candidates for this failure: (1) mice vaccinated at week 2, when a large tumor burden has been established, can still recruit a high number of effector CTLs, but suppression of CTL activity by either the tumor itself or by tumor-induced suppressor cells leads to unfavorable clinical outcome; (2) mice vaccinated at week 2 may finally fail to eliminate leukemia, because the tumor-specific CTL clone develops increased susceptibility to "clonal exhaustion" (activation-induced cell death) as a result of heavy antigenic stimulation by the large tumor burden.⁵⁴ It should be mentioned here that the reported⁵⁵ slow Th2 effectors' death as opposed to the rapid Th1 effectors' death may account for the Th2 cytokine profile observed in cases with advanced disease.^{26,27}

In conclusion, our data demonstrate that in this AML model, tumor growth does not induce tolerance or an altered Th2 type cytokine profile that might impede a cytotoxic T-cell-mediated immune response. Moreover, our results demonstrate that reduction of the tumor load with chemotherapy leads to more effective B7 vaccines, but it still needs to be determined which immune mechanisms regulate the final outcome of the vaccines and how tumor burden interferes with them. It is conceivable that in diseases like AML clinical trials based on genetically modified tumor cell vaccines will target residual disease after treatment either with chemotherapy or bone marrow transplantation. It is therefore clinically important that B7 vaccines not only initiate an antitumor effector T-cell response in these patients, but also sustain it and successfully lead to development of tumor-specific memory T cells able to "fight" tumor cell recurrence. To date, very little is known on the development from naive to tumor-specific memory T cells, and clearly more research in this direction is needed.

ACKNOWLEDGMENT

The authors thank John Delmonte Jr and Mahesh Karandikar for expert technical assistance.

REFERENCES

1. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A, Boon T: A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 254:1643, 1991
2. Urban JL, Schreiber H: Tumor-antigens. *Annu Rev Immunol* 10:617, 1992
3. Boon T, Cerottini J-C, Van den Eynde B, Van der Bruggen P, Van Pel A: Tumor-antigens recognized by T lymphocytes. *Annu Rev Immunol* 12:337, 1994
4. Kawakami Y, Eliyahu S, Delgado CH, Robbins PF, Rivoltini L, Topalian SL, Miki T, Rosenberg SA: Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. *PNAS* 91:3515, 1994
5. Kawakami Y, Eliyahu S, Jennings C, Sakaguchi K, Kang X, Southwood S, Robbins PF, Sette A, Appella E, Rosenberg SA: Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with in vivo tumor regression. *J Immunol* 154:3961, 1995
6. Robbins PF, el-Gamil M, Li YF, Topalian SL, Rivoltini L, Sakaguchi K, Appella E, Kawakami Y, Rosenberg SA: Cloning of a new gene encoding an antigen recognized by melanoma-specific

HLA-A24-restricted tumor-infiltrating lymphocytes. *J Immunol* 154:5944, 1995

7. Van Pel A, Van der Bruggen P, Coulie PG, Brichard VG, Lethe B, Van den Eynde B, Uyttenhove C, Renauld J-C, Boon T: Genes coding for tumor-antigens recognized by cytolytic lymphocytes. *Immunol Rev* 145:229, 1995

8. Henderson RA, Finn OJ: Human tumor antigens are ready to fly. *Adv Immunol* 62:217, 1996

9. Schwartz RH: Costimulation of T lymphocytes: The role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. *Cell* 71:1065, 1992

10. Gajewski TF, Fallarino F, Uyttenhove C, Boon T: Tumor rejection requires a CTLA4 ligand provided by the host or expressed on the tumor. *J Immunol* 156:2909, 1996

11. Colombo MP, Forni G: Cytokine gene transfer in tumor inhibition and tumor therapy: Where are we now? *Immunol Today* 15:48, 1994

12. Dranoff G, Mulligan RC: Gene transfer as cancer therapy. *Adv Immunol* 58:417, 1995

13. Pardoll DM: Paracrine cytokine adjuvants in cancer immunotherapy. *Annu Rev Immunol* 13:399, 1995

14. Liu Y, Linsley PS: Costimulation of T-cell growth. *Curr Opin Immunol* 4:265, 1992

15. Chen L, Linsley PS, Hellström KE: Costimulation of T cells for tumor immunity. *Immunol Today* 14:483, 1993

16. Linsley PS, Ledbetter JA: The role of the CD28 receptor during T cell responses to antigen. *Annu Rev Immunol* 11:191, 1993

17. June CH, Bluestone JA, Nadler LM, Thompson CB: The B7 and CD28 receptor families. *Immunol Today* 15:321, 1994

18. Chen L, Ashe S, Brady WA, Hellström I, Hellström KE, Ledbetter JA, McGowan P, Linsley P: Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell* 71:1093, 1992

19. Townsend SE, Allison JP: Tumor rejection after direct costimulation of CD8⁺ T cells by B7-transfected melanoma cells. *Science* 259:368, 1993

20. Baskar S, Ostrand-Rosenberg S, Nabavi N, Nadler LN, Freeman GJ, Glimcher LH: Constitutive expression of B7 restores immunogenicity of tumor cells expressing truncated major histocompatibility complex class II molecules. *Proc Natl Acad Sci USA* 90:5687, 1993

21. Hodge JW, Abrams S, Schlom J, Kantor JA: Induction of antitumor immunity by recombinant vaccinia viruses expressing B7.1 or B7.2 costimulatory molecules. *Cancer Res* 54:5552, 1994

22. Matulis UA, Dosiou C, Lamont C, Freeman GJ, Mauch P, Nadler LM, Griffin JD: Role of B7.1 mediating an immune response to myeloid leukemia cells. *Blood* 85:2507, 1995

23. Dunussi-Joannopoulos K, Weinstein HJ, Nickerson PW, Strom TB, Burakoff SJ, Croop JM, Arceci RJ: Irradiated B7.1 transduced primary acute myelogenous leukemia (AML) cells can be used as therapeutic vaccines in murine AML. *Blood* 87:2938, 1996

24. Iwahashi M, Tanimura H, Yamaue H, Tsunoda T, Tani M, Tamai M, Noguchi K, Hotta T: Defective autologous mixed lymphocyte reaction (AMLR) and killer activity generated in the AMLR in cancer patients. *Int J Cancer* 51:67, 1992

25. Ochoa AC, Longo DL: Alteration of signal transduction in T cells from cancer patients, in De Vita VT, Hellman S, Rosenberg SA (eds): *Important Advances in Oncology*. Philadelphia, PA, Lippincott, 1995, p 43

26. Schoof DD, Terashima Y, Peoples GE, Goedegebuure PS, Andrews JV, Richie JP, Eberlein TJ: CD4⁺ T cell clones isolated from human renal cell carcinoma possess the functional characteristics of Th2 helper cells. *Cell Immunol* 150:114, 1993

27. Ghosh P, Komschlies KL, Cipitelli M, Longo DL, Subleski

J, Ye J, Sica A, Young HA, Wiltout RH, Ochoa AC: Gradual loss of T-helper 1 population in spleen of mice during progressive tumor growth. *J Natl Cancer Inst* 87:1478, 1995

28. Zeh HJ, Hurd S, Storkus WJ, Lotze MT: Interleukin-12 promotes the proliferation and cytolytic maturation of immune effectors: Implications for the immunotherapy in cancer. *J Immunother* 14:155, 1993

29. Cardoso AA, Schultze JL, Boussiotis VA, Freeman GJ, Seamon MJ, Laszlo S, Billet A, Sallan SE, Gribben JG, Nadler LM: Pre-B acute lymphoblastic leukemia cells may induce T-cell anergy to alloantigen. *Blood* 88:41, 1996

30. Resnitzky P, Estrov Z, Haran-Ghera N: High incidence of acute myeloid leukemia in SJL/J mice after x-irradiation and corticosteroids. *Leuk Res* 9:1519, 1985

31. Marshak-Rothstein A, Fink P, Gridley T, Raulet DH, Bevan MJ, Gefer ML: Properties and applications of monoclonal antibodies directed against determinants of the Thy-1 locus. *J Immunol* 122:2491, 1979

32. Spitalny G, Havell E: Monoclonal antibody to murine gamma interferon inhibits lymphokine-induced antiviral and macrophage tumoricidal activities. *J Exp Med* 159:1560, 1984

33. Cherwinski HM, Schumacher JH, Brown KD, Mosmann TR: Two types of mouse helper T cell clones: III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. *J Exp Med* 166:1229, 1987

34. Ohara J, Paul WE: Production of a monoclonal antibody to and molecular characterization of B-cell stimulatory factor-1. *Nature* 315:33, 1985

35. Abrams JS, Roncarolo MG, Yssel H, Andersson U, Gleich GJ, Silver JE: Strategies of anti-cytokine monoclonal antibody development: Immunoassay of IL-10 and IL-5 in clinical samples. *Immunol Rev* 127:5, 1992

36. Mosmann TR, Coffman RL: Th1 and Th2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 7:145, 1989

37. Mosmann TR, Sad S: The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today* 17:138, 1996

38. Wierenga EA, Snoek M, de Groot C, Chretien I, Bos JD, Jansen HM, Kapsenberg ML: Evidence for compartmentalization of functional subsets of CD2⁺ T lymphocytes in atopic patients. *J Immunol* 144:4651, 1990

39. Seder RA, Paul WE: Acquisition of lymphokine-producing phenotype by CD4⁺ T cells. *Ann Rev Immunol* 12:635, 1994

40. Mossmann TR, Moore KW: The role of IL-10 crossregulation of Th1 and Th2 responses. *Immunol Today* 12: A49, 1994

41. Ben-Ishay Z, Prindull G, Sharon S: Improved prognosis in mice with advanced myeloid leukemia following administration of GM-CSF and cytosine arabinoside. *Leuk Res* 15:321, 1991

42. Mueller DL, Jenkins MK, Schwarz RH: Clonal expansion versus functional clonal inactivation: A costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Ann Rev Immunol* 7:445, 1989

43. Schwarz RH: A cell culture model for T lymphocyte clonal anergy. *Science* 248:1349, 1990

44. Li W, Whaley CD, Mondino A, Mueller DL: Blocked signal transduction to the ERK and JNK protein kinases in anergic CD4⁺ T cells. *Science* 271:1272, 1996

45. Migita K, Eguchi K, Kawabe Y, Tsukada T, Ichinose Y, Nagataki S: Defective TcR-mediated signaling in anergic T cells. *J Immunol* 155:5083, 1995

46. Kersh GJ, Allen PM: Essential flexibility in the T-cell recognition of antigen. *Nature* 380:495, 1996

47. Huang AYC, Golumbek P, Ahmadzadeh M, Jaffe E, Pardoll

M, Levitsky H: Role of bone-marrow-derived cells in presenting MHC class I-restricted tumor antigens. *Science* 264:961, 1994

48. Huang AYC, Bruce AT, Pardoll DM, Levitsky HI: Does B7.1 expression confer antigen-presenting cell capacity to tumors in vivo? *J Exp Med* 183:769, 1996

49. Mayordomo JI, Zorina T, Storkus WJ, Zitvogel L, Celluzzi C, Falo LD, Melief CJ, Ildstad ST, Kast WM, Deleo AB, Lotze MT: Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumour immunity. *Nature Med* 1:1297, 1995

50. Viola A, Lanzavecchia A: T cell activation determined by T cell receptor number and tunable thresholds. *Science* 273:104, 1996

51. Lanzavecchia A: T cell clones retained small numbers of TcRs for as long as 5 to 8 days after stimulation, in Matzinger P, Flaginek

M, Rammensee H-G, Storkinger G, Rolink T, Nicklin L (eds): *The Tolerance Workshop 1986*. Basel, Switzerland, Roche, 1986, p 293

52. Chen L, McGowan P, Ashe S, Johnston JV, Hellström I, Hellström KE: B7.1/CD80 transduced tumor cells elicit better systemic immunity than wild-type tumor cells admixed with *Corynebacterium parvum*. *Cancer Res* 54:5420, 1994

53. Chen L, McGowan P, Ashe S, Johnston JV, Li Y, Hellström I, Hellström KE: Tumor immunogenicity determines the effect of B7 costimulation on T cell-mediated tumor immunity. *J Exp Med* 179:523, 1994

54. Ahmed R, Gray D: Immunological memory and protective immunity: Understanding their relation. *Science* 272:54, 1996

55. Swain SL, Croft M, Dubey C, Haynes L, Rogers P, Zhang X, Bradley LM: From naive to memory cells. *Immunol Rev* 150:143, 1996



Potent Activity of Soluble B7-IgG Fusion Proteins in Therapy of Established Tumors and as Vaccine Adjuvant

Knut Sturmhoefel,¹ Kwang Lee, Gary S. Gray, Jenifer Thomas, Richard Zollner, Margot O'Toole, Holly Swiniarski, Andrew Dörner, and Stanley F. Wolf

Genetics Institute, Andover, Massachusetts 01810

ABSTRACT

Fusion proteins consisting of the extracellular region of murine B7.1 or B7.2 and the Fc portion of murine IgG2a (B7-IgG) were evaluated for their ability to promote antitumor responses. Therapeutic administration of soluble B7-IgG in mice with established tumors induced complete regression of the tumor and increased the survival of mice. In three models, MethA, P815, and MB49, mice with 7-day-old established tumors were cured with two to three treatment cycles of B7-IgG, given twice a week. Even in mice with an established B16/F10 tumor (a poorly immunogenic melanoma), therapeutic treatment with B7-IgG alone slowed tumor growth and increased survival significantly. Still stronger antitumor activity was achieved when B7-IgG was used as a vaccine adjuvant mixed with irradiated tumor cells. In 80% of mice with 7-day-old B16 tumors, tumors regressed completely, and mice survived for at least 80 days. In all tumor models, B7.1-IgG and B7.2-IgG had similar antitumor activity. B7-IgG-mediated tumor rejection was dependent on T cells, specifically CD8 cells, as demonstrated by the failure of B7-IgG to induce tumor regression in severe combined immunodeficient or CD8-depleted mice. In addition, mice that were cured of an established tumor were protected against a rechallenge with the same tumor for at least 4 months, suggesting the generation of memory responses. Surprisingly, the antitumor activity of B7-IgG was independent of IFN- γ , as demonstrated by tumor rejection in IFN- γ knockout mice. Our findings demonstrate the potent capacity of B7-IgG to generate or enhance antitumor immune responses and suggest the clinical value of B7-IgG.

INTRODUCTION

Optimal activation of naïve T cells requires signaling through the T-cell receptor as well as through other costimulatory pathways. Naïve T cells stimulated only through the T-cell receptor become nonresponsive, anergic, or die. One such costimulatory signal can be provided by the interaction of B7.1 or B7.2 on APCs² with CD28 on T cells (1–4). However, binding of B7.1 or B7.2 to another receptor on activated T cells, CTLA-4, has been shown to down-regulate immune responses. CTLA-4, a homologue of CD28, binds B7.1 and B7.2 with higher affinity than CD28 and is only expressed on activated T cells. It has been proposed that binding of B7 to CTLA-4 counterbalances CD28-mediated stimulation (1, 2, 5).

Established tumors in mice can be cured by immune-mediated mechanisms (6–8). However, it is also clear that tumors can escape a developing immune response by several mechanisms (7, 9–13). Ineffective activation of T cells due to the absence of appropriate costimulation provides one explanation for inadequate immune responses to growing tumors. It has been proposed that tumor-associated antigens are presented to T cells by nonprofessional APCs lacking costimulatory signals. This suboptimal stimulation leads to

anergy or tolerance of tumor-specific T cells (14–17). Several investigators have had some success in enhancing tumor-specific T-cell activation and/or preventing anergy or tolerance by expressing costimulatory molecules on the surface of tumor cells. Vaccination of mice with tumor cells transfected with B7.1 or B7.2 cDNA protected mice against tumor challenge (18) and induced regression of established tumors in some therapeutic models (17, 19–25). Similarly, direct injection of B7-expressing DNA vectors into tumor-bearing mice has enhanced immune responses and promoted tumor rejection (26). Blocking the interaction of B7 with CTLA-4, thereby preventing negative signals triggered by CTLA-4, has also been used as an approach to enhance antitumor activity (27, 28).

In what may be a more clinically applicable approach to provide B7/CD28-mediated costimulation, we have developed soluble B7-IgG fusion proteins and tested their efficacy in several therapeutic tumor models and protocols. *In vitro* studies with these fusion proteins have demonstrated their costimulatory activity in enhancing proliferation and cytokine production from naïve T cells (29).³ *In vivo* studies using B7.2-IgG as a vaccine adjuvant have demonstrated its capacity to enhance the generation of proliferative and CTL responses to peptide vaccines (30). In the studies reported here, we show that B7.1-IgG or B7.2-IgG mixed with irradiated tumor cell vaccines or administered alone has potent antitumor activity, leading to the regression and cure of established tumors or significantly increased survival of tumor-bearing mice.

MATERIALS AND METHODS

Mice

Female 6–8-week-old BALB/c, C57BL/6, and DBA/2 mice were purchased from either Taconic (Germantown, NY) or The Jackson Laboratory (Bar Harbor, ME). The 6–8-week-old BALB/c-IFN- γ knockout mice were purchased from The Jackson Laboratory. Mice were housed under pathogen-free conditions at Genetics Institute (Andover, MA).

Monoclonal Abs

Rat antimouse CD4 and CD8 monoclonal Abs (GK1.5 and 53-6.72; American Type Culture Collection, Manassas, VA) were produced and purified by standard techniques at Genetics Institute.

B7-IgG Fusion Proteins

Expression plasmids encoding murine B7.1 or B7.2 signal and extracellular domains fused to the Fc region of murine IgG2a were constructed as follows: cDNAs encoding the signal and extracellular domains of murine B7.1 and B7.2 were generated by PCR amplification from cloned cDNA (31, 32). For B7.1, the cDNA extends from the initiation Met in the signal sequence through Asp²⁴³ of the total protein sequence [i.e., Met-Ala-Cys-Asp-Cys . . . Glu-Asp-Pro-Pro-Asp (31)]. For B7.2, the cDNA extends from the initiation Met in the signal sequence through Pro²³⁹ of the total protein sequence [i.e., Met-Asp-Pro-Arg-Cys . . . Glu-Phe-Pro-Ser-Pro (32)]. The B7 sequences were joined to a genomic DNA segment encoding the hinge-CH2-CH3 domains for a murine IgG2a Ab (the protein sequence was identical to that of GenBank accession

Received 4/7/99; accepted 7/30/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ To whom requests for reprints should be addressed. Present address: Lexigen Pharmaceuticals, 125 Hartwell Avenue, Lexington, MA 02421-3125. Phone: (781) 861-5300, ext. 235; Fax: (781) 861-5301; E-mail: Knut.Sturmhoefel@emindustries.com.

² The abbreviations used are: APC, antigen-presenting cell; i.f.p., intra-footpad; i.d., intradermal; FcR, Fc receptor; Ab, antibody; SCID, severe combined immunodeficient; CHO, Chinese hamster ovary; FACS, fluorescence-activated cell-sorting.

³ Unpublished results.

number J00470) such that the IgG2a sequence begins with Glu-Pro-Arg-Gly and ends with Ser-Arg-Thr-Pro. The Cys residues within the Ab hinge region were retained such that the two B7-hinge-CH2-CH3 chains were covalently linked. We also generated fusion proteins (designated B7.1-IgG2mut or B7.2-IgG2mut) in which the IgG2a regions were mutated to ablate binding to Fc- γ RI and complement C1q. The following amino acid residues in the CH2 domain were replaced by Ala: (a) Leu²³⁵; (b) Glu³¹⁸; (c) Lys³²⁰; and (d) Lys³²² (33).

For production of B7-IgG proteins, the reconstructed sequences were inserted in the pHTOP expression vector (34). The recombinant plasmids were transfected into the CHO cell line and amplified by standard techniques (34). CHO cells expressing B7-IgG were grown in DME/F12 (Life Technologies, Inc.) supplemented with 10% FCS, 0.02 μ M methotrexate (34), and 1 mg/ml G418 (Geneticin; Life Technologies, Inc.). At confluence, growth media were discarded, the cells were washed with PBS, and serum-free medium was added. Culture supernatants were collected at 24 h, clarified by sequential passage through 5.0 and 0.22 μ m filters, and concentrated using a 30-kDa tangential flow cartridge filter. The concentrate was loaded onto a protein A-Sepharose Fast Flow column (Pharmacia Biotech), washed with PBS, and eluted with 20 mM citrate (pH 3.0). Elution fractions containing the fusion protein were neutralized with 1 M Tris (pH 8.0; Sigma, St. Louis, MO), and the material was formulated in PBS (pH 7.2) by buffer exchange using a stirred cell with YM30 membrane (Amicon, Beverly, MA). Protein was depyrogenated by chromatography on Poros PI (PerSeptive Biosystems, Framingham, MA). Protein concentration was calculated using an absorbance at 280 nm and a theoretical extinction coefficient of 1.33 cm/mg·ml. More than 99% of the protein was in the dimeric, nonaggregated form, as determined by a TSK 3000 SWXL column [TosoHaas USA, Montgomeryville, PA; PBS (pH 6.8) running buffer]. Endotoxin levels were less than 0.25 endotoxin unit/mg as determined by gel clot assay (Cape Cod Associates).

Tumor Models

All tumor cell lines were cultured in DMEM supplemented with 10% FCS (Sigma) without antibiotics. The following tumor cell lines were used: (a) MethA sarcoma (35); (b) B16/F10 melanoma (36); and (c) MB49 bladder carcinoma (37); P815 and P815-B7.1 were generously provided by T. Gajewski (University of Chicago, Chicago, IL; Refs. 38 and 39). Expression of the transfected mB7.1 in P815-B7.1 was verified by FACS analysis, and the mean fluorescence intensity of 100% of cells was 2.5 log above the unstained control. Solid tumors were established by i.d. or s.c. injection of tumor cells in the flank of the appropriate mouse strain. Five $\times 10^4$ P815 cells were injected into DBA/2 mice; 5×10^5 MethA cells were injected into BALB/c mice; and 2×10^5 B16/F10 cells or 1×10^5 MB49 cells were injected into C57BL/6 mice. Tumor-bearing mice either died within 20–35 days after tumor inoculation (spontaneously metastasizing P815 and B16/F10 tumors) or were sacrificed when the tumors reached a size of approximately 360–400 mm². For purposes of graphic representation of tumor growth, animals that died or had to be sacrificed were assigned a tumor size of 400 mm².

Vaccination Protocols

Prophylactic Protocol. Mice were immunized on day 0 with 1×10^7 irradiated tumor cells in PBS alone or mixed with 75–100 μ g of murine B7.1-IgG, murine B7.2-IgG, or murine IgG. Injections were administered i.f.p. in both hind legs. Mice were also treated with B7-IgG or murine IgG alone on day 5 and challenged on day 7 by i.d. injection in the right flank with live tumor cells (cell number as described above, in 50 μ l).

Therapeutic Protocol. A primary tumor was established by i.d. injection as described above. On day 7–9, when tumors were palpable, mice were vaccinated i.f.p. with 5×10^5 irradiated tumor cells mixed with 25–100 μ g of B7.1-IgG, B7.2-IgG, vehicle (PBS), or irrelevant isotype-matched Ab. Additional B7-IgG, IgG, or vehicle alone was injected i.f.p. 3 days later. This vaccination regimen was repeated weekly for 2–6 weeks.

Therapy with B7-IgG alone was used to treat mice bearing 7-day-old tumors with 25–100 μ g of B7.1-IgG or B7.2-IgG. Injections were i.f.p. twice a week for 2–3 weeks.

Cell Depletions

CD4 or CD8 T cells were depleted by i.p. injections of 100–150 μ g of monoclonal Ab GK1.5 or 53-6.72. Ab treatment was started on day 6, 1 day before initiation of B7-IgG therapy, and continued on days 7, 8, 10, 14, 17, and 21 after tumor inoculation. CD4 or CD8 T-cell depletion was verified by FACS analysis from peripheral blood lymphocytes.

Statistical Analysis

Survival curves were analyzed by the Kaplan-Meier method using the statistical analysis software JMP (version 3.1 for Macintosh; SAS Institute Inc., Cary, NC) according to the manufacturer's specifications.

RESULTS

B7.1-IgG or B7.2-IgG Enhances the Protective Efficacy of an Irradiated Tumor Cell Vaccine. Preliminary studies established that the fusion proteins were effective for costimulation *in vitro* if they were plate-bound, and their activity could be blocked by soluble anti-B7.1 or B7.2 Ab, but not by soluble anti-CTLA-4 Ab (data not shown). These data suggest that, *in vitro*, immobilized B7-IgG provides a costimulatory signal by cross-linking CD28.

To assess *in vivo* function, we first evaluated the activity of B7-IgG fusion proteins in prophylactic tumor vaccine models. Naïve mice were vaccinated with irradiated P815 tumor cells alone or with irradiated cells mixed with B7.1-IgG or B7.2-IgG as described in "Materials and Methods." If included, B7-IgG alone was given again on day 5. Mice were challenged on day 7 with live P815 cells. By day 10, all mice that had not been vaccinated or had been vaccinated with irradiated cells alone developed solid tumors (Table 1). In contrast, 60–70% of mice vaccinated with a combination of irradiated tumor cells and B7.1-IgG or B7.2-IgG were protected against the tumor challenge as assessed by the absence of palpable tumors at day 21. Similar results were obtained in the MethA and B16/F10 tumor models (data not shown).

Our *in vitro* data suggested that aggregation or cross-linking of the B7-IgG proteins was required for costimulation. Therefore, we evaluated the role of the IgG domain and FcR binding in the *in vivo* function of the fusion proteins. Mice were vaccinated with irradiated P815 tumor cells mixed with fusion proteins mutated in the CH2 FcR-binding region (B7.1-IgGmut and B7.2-IgGmut). These mutations are reported to ablate binding to Fc- γ RI receptors and complement (33). In the P815 prophylactic model, the mutated molecules were less effective than the wild-type fusion proteins (Table 1), although both forms exhibited similar costimulatory activity *in vitro* when plate-bound or cross-linked by an antimurine IgG Ab (data not shown). These findings suggest that *in vivo*, in prophylactic models,

Table 1 Protection against tumor challenge after prophylactic vaccination with irradiated tumor cells mixed with B7-IgGs

DBA/2 mice were immunized as described in "Materials and Methods" with irradiated tumor cells mixed with B7-IgG or treated with B7-IgG alone. In all experiments, mice were challenged with 2×10^5 live P815 tumor cells on day 7. Protection against the challenge was determined by the absence of a palpable tumor after 21 days. Protection is shown as the mean percentage (\pm SD) of tumor-free mice from one to five independent experiments.

Immunization	% Mean protection (\pm SD)	No. of experiments
None	8 (11)	5
Irradiated P815	2 (4)	5
Irradiated P815 + B7.1-IgG	65 (18)	4
Irradiated P815 + B7.2-IgG	67 (22)	5
B7.1-IgG alone	13 (0)	2
B7.2-IgG alone	9 (11)	4
Irradiated P815-B7.1 transfectant	23 (4)	2
Irradiated P815 + B7.1-IgG mutated	28 (4)	2
Irradiated P815 + B7.2-IgG mutated	20	1

Fc binding of the B7-IgG molecules is important for costimulating unprimed T cells.

Many groups have reported that vaccines of tumor cells expressing membrane-bound B7.1 or B7.2 generate protective antitumor immunity (17–25). We compared the vaccine efficacy of irradiated P815 tumor cells that had been transfected with and expressed high levels of B7.1 (P815-B7.1) with irradiated wild-type P815 cells mixed with B7.1-IgG or B7.2-IgG. Vaccination with irradiated P815-B7.1 cells protected 23% of mice. In contrast, vaccination with irradiated wild-type P815 cells mixed with soluble B7.1-IgG or B7.2-IgG protected 65% and 67% of mice, respectively (Table 1). Similar results were obtained when comparing B16/F10-B7.1 transfectants with B16/F10

cells mixed with the fusion proteins (data not shown). These observations demonstrate the efficacy of soluble B7.1-IgG and B7.2-IgG as vaccine adjuvant and indicate that they may be more potent than tumor cell transfectants expressing high levels of membrane-bound B7.1.

Therapeutic Vaccination with Irradiated P815 Tumor Cells Mixed with B7.1-IgG or B7.2-IgG Cures Mice of Established P815 Tumor. To test the adjuvant activity of B7-IgG in a therapeutic tumor vaccine model, DBA/2 mice with 7-day-old s.c. P815 tumors were injected i.f.p. with irradiated P815 tumor cells alone or mixed with B7.1-IgG or B7.2-IgG. A second dose of B7-IgG alone was administered i.f.p. 3–5 days later. This treatment was repeated weekly for 3

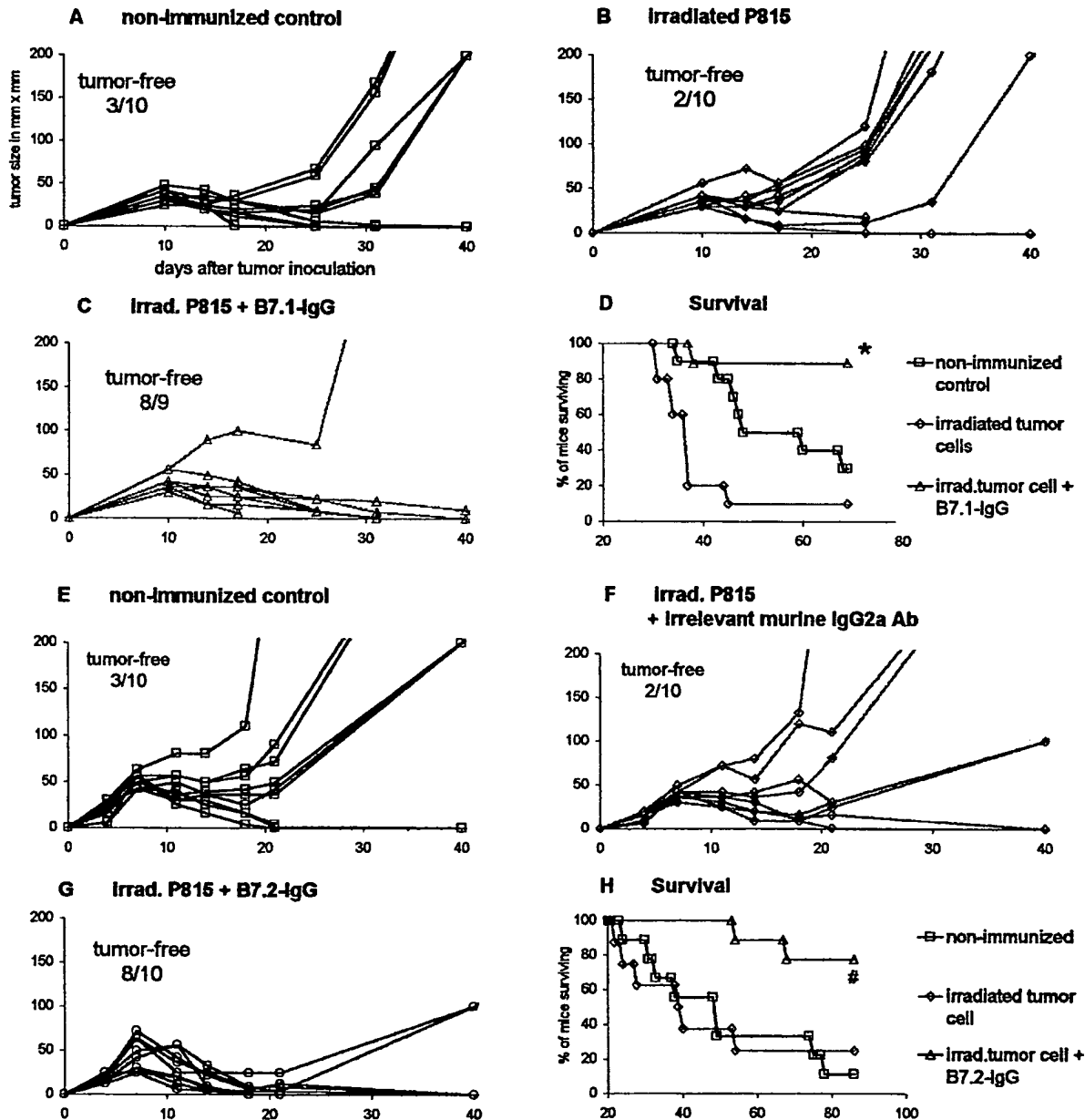


Fig. 1. Vaccination of P815 tumor-bearing mice with irradiated P815 tumor cells mixed with B7.1-IgG or B7.2-IgG induces tumor regression and prolongs survival. P815 tumors were established on day 0 by i.d. injection of 5×10^4 P815 cells. Mice were vaccinated on day 7 by i.f.p. injection with either PBS (A and E), irradiated P815 tumor cells (B), tumor cells mixed with irrelevant mouse IgG2a Ab (F), or irradiated P815 cells mixed with B7.1-IgG or B7.2-IgG (C and G). PBS, irrelevant Ab, or the same B7-IgG as used initially was readministered 3 days later. The vaccination cycle was repeated for 3 weeks. Tumor growth was monitored, and the number of tumor-free mice after 40 days is indicated. In A, B, C, E, F, and G, each line represents the tumor growth of an individual mouse. Mice that were euthanized or died of metastatic disease were assigned a tumor size of 400 mm². D and H show the percentage of surviving animals/group as a function of time. Survival of mice treated with B7.1-IgG or B7.2-IgG was significantly increased compared with the controls (*, $P < 0.017$; #, $P < 0.0027$, respectively, by log-rank test). The data are representative of four independent experiments.

weeks. Beginning about 1 week after the first immunization, reduced tumor growth and tumor regression were observed in mice treated with tumor cells mixed with either of the B7-IgGs (Fig. 1, C and G). Tumor growth was not reduced in most mice treated with irradiated tumor cells alone or with cells mixed with an irrelevant mouse IgG2a Ab (Fig. 1, B and F). In five independent experiments, primary tumors disappeared in 60–90% of mice treated for three cycles with irradiated P815 tumor cells mixed with either B7.1-IgG or B7.2-IgG, compared with 10–30% in the control groups. Regression of the primary tumor also correlated with statistically significant increases in survival (Fig. 1, D and H).

B7-IgG as Therapeutic Tumor Vaccine Adjuvant in Different Tumor Models. To demonstrate that the results with B7-IgG were not unique to the P815 tumor or the DBA/2 mouse strain, we tested the efficacy of B7-IgG as an adjuvant in three additional tumor models in two other mouse strains. BALB/c mice bearing 7-day-old s.c. MethA sarcomas were treated with PBS or irradiated MethA cells alone or mixed with either B7.1-IgG or B7.2-IgG. Two vaccinations with irradiated MethA cells mixed with B7-IgG led to complete tumor regression in 90–100% of mice (Fig. 2, C and D). Mice were tumor free 60–90 days later. In groups left untreated, treated with irradiated MethA cells alone (Fig. 2, A and B), or treated with irradiated cells mixed with an irrelevant mouse IgG2a (two independent studies; data not shown) tumors regressed in only 10% of mice. We obtained comparable results with MB49 tumor cells in C57BL/6 mice (Fig. 2, E–H).

In the poorly immunogenic melanoma model, B16/F10 (in C57BL/6 mice), therapeutic vaccination with irradiated tumor cells and B7-IgG proteins significantly reduced tumor growth and increased long-term survival (Fig. 2, I–L). By day 35, all mice left untreated or vaccinated with irradiated cells alone had succumbed to the tumor or were euthanized with large tumor masses (Fig. 2, I, J, and L). In contrast, 60% of mice immunized with the B7.1-IgG vaccine (data not shown) and 90% of mice immunized with the B7.2-IgG vaccine survived for >80 days (Fig. 2, K and L).

Therapeutic Administration of B7-IgG Alone Induces Antitumor Responses. The potent adjuvant activity of B7.1-IgG and B7.2-IgG in the therapeutic vaccine models prompted us to test their efficacy as therapeutic agents alone. In all four therapeutic tumor models, treatment with B7.1-IgG or B7.2-IgG alone reduced tumor growth and increased survival (Fig. 3). In three of the models, the efficacy of treatment with B7-IgG alone was similar to that of vaccination with irradiated cells mixed with B7-IgG (Fig. 3, A–C). In the B16/F10 model, treatment with B7-IgG alone slowed tumor growth and significantly increased survival time. However, tumor cure in 80% of B16/F10 tumor-bearing mice could only be achieved by vaccination with irradiated tumor cells and B7.1-IgG or B7.2-IgG as adjuvant (Fig. 3D). The B16/F10 tumor was also the only model in which B7.2-IgG was slightly more effective than B7.1-IgG. In all other models, B7.1-IgG and B7.2-IgG had similar activity.

In contrast to the prophylactic vaccine models, where efficacy was reduced for fusion proteins mutated in the Fc binding domain, therapeutic vaccination with the mutated forms of B7-IgG was as efficacious as treatment with the wild-type forms (data not shown). These data suggest that the activity of B7-IgG in a therapeutic setting does not require FcR binding and demonstrate the potency of B7-IgG as antitumor agent.

B7-IgG-mediated Tumor Cure Is CD8 but not CD4 T-Cell Dependent and Generates Lasting Protective Immunity. To determine whether the antitumor response mediated by B7-IgG is dependent of the adaptive immune response, we evaluated B7-IgG therapy in SCID mice. BALB/c-SCID mice bearing MethA tumors were treated with B7.2-IgG alone or mixed with irradiated MethA cells.

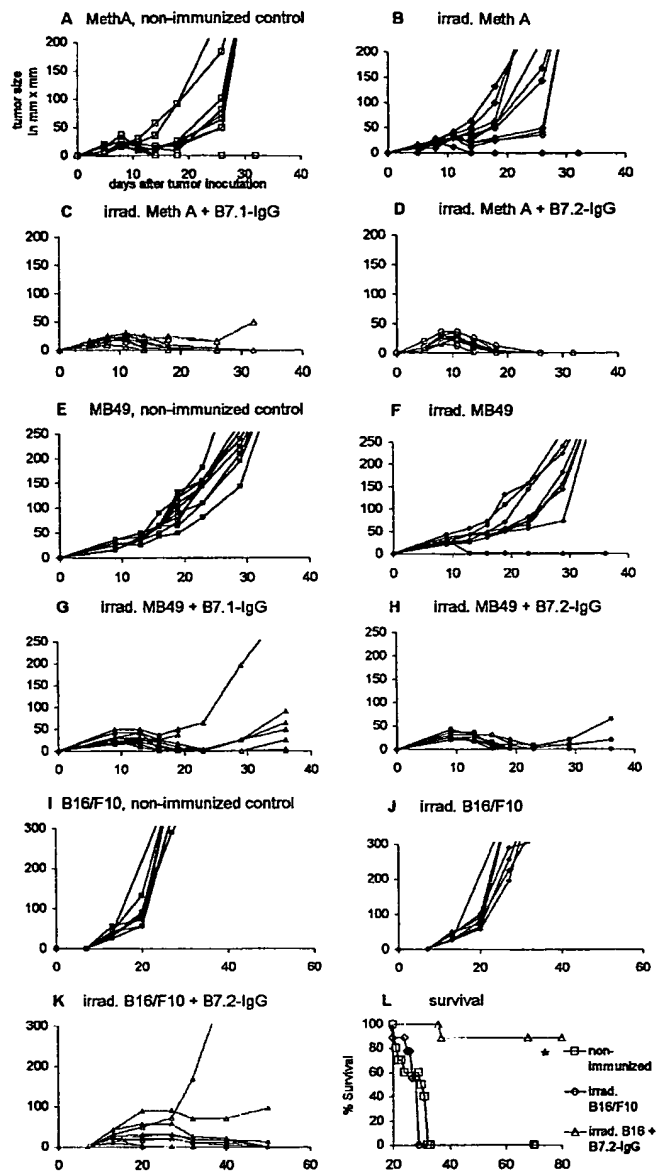


Fig. 2. Vaccination with B7-IgG as an adjuvant is effective in three other therapeutic tumor models. Solid tumors were established on day 0 as described in "Materials and Methods." A total of 8–10 mice/group were vaccinated, starting on day 7, by i.p. injection with either irradiated tumor cells (B, F, and I) or cells mixed with 25 (C and D), 50 (G and H), or 100 μ g (K) of B7.1-IgG or B7.2-IgG, respectively. PBS, B7.1-IgG, or B7.2-IgG, as used in the initial injection, was given again 3–4 days later. One group was treated with PBS alone (A and E; data not shown for B16/F10; IgG2a isotype controls were included in two experiments). Tumor growth was monitored for 35–60 days. Mice with MethA or MB49 tumors were euthanized once the tumor reached a size of about 360 mm². B16/F10-bearing mice either died from metastatic disease or were euthanized when their tumors reached about 360 mm². In A–K, each line represents the tumor growth of an individual mouse. The percentage of surviving animals/group is shown in L (*, $P < 0.0001$ by log-rank test). Experiments were repeated two to five times with comparable results.

Neither treatment had an effect on tumor growth (Fig. 4), proving the dependence of B7-IgG-mediated tumor responses on T or B cells. To further define the T-cell subsets important for the antitumor activity of B7-IgG, we treated MethA tumor-bearing mice after depleting CD8 or CD4 T cells. Depletion of CD8 or CD4 T cells by Ab injection was started 6 days after tumor inoculation, i.e., 1 day before initiation of B7-IgG therapy. Successful depletion was verified by FACS analysis of peripheral blood lymphocytes on day 28: CD4 cells were undetectable; and CD8 cells were <1.5% of peripheral blood lymphocytes. In

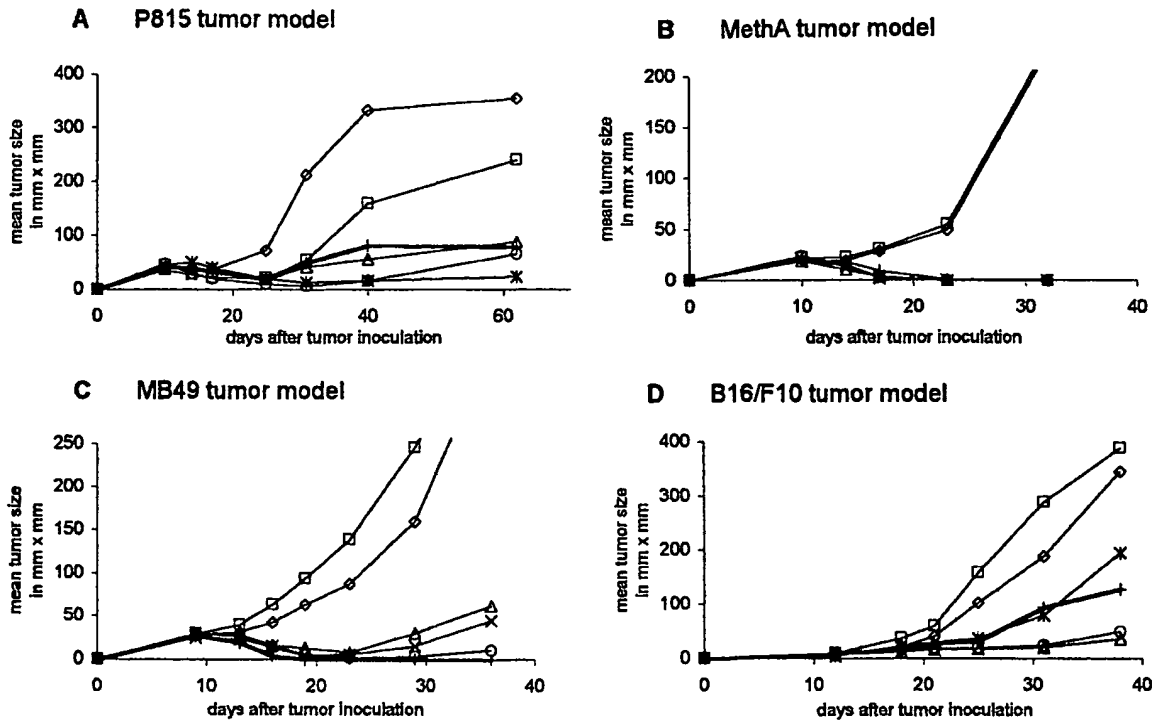


Fig. 3. Therapeutic administration of B7-IgG alone has antitumor activity. Mice were inoculated on day 0 with live P815 (A), MethA (B), MB49 (C), or B16/F10 (D) tumor cells as described in "Materials and Methods." Treatment started on days 6–8. Mice were treated with PBS (□), irradiated tumor cells alone (◇), irradiated tumor cells mixed with B7.1-IgG (Δ), or B7.2-IgG (○) or with B7.1-IgG (*) or B7.2-IgG alone (+). The mean tumor size/group (groups of 7–10 mice) is plotted. Mice were euthanized when tumor size reached 360 mm², and mice that died of metastatic disease were assigned a tumor size of 400 mm².

CD4-depleted mice, the growth of MethA tumors was comparable with that in untreated normal mice (Fig. 5, A and B). Therapy with B7.2-IgG induced complete tumor regression and cure in CD4-depleted mice (Fig. 5C). In contrast, treatment with B7.2-IgG in CD8-

depleted mice slowed tumor growth but did not cure the mice (Fig. 5, D and E). Thus, depletion of CD8 T cells abrogated the antitumor activity of B7.2-IgG.

Further evidence that B7-IgG therapy of established tumors de-

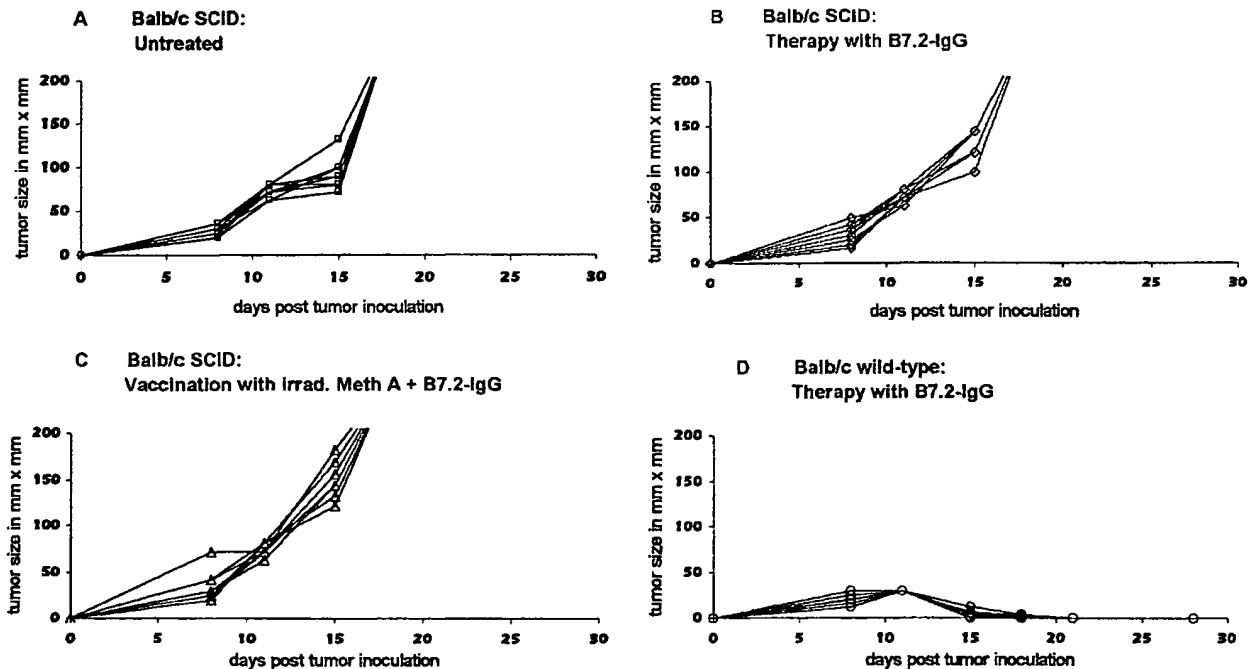


Fig. 4. B7-IgG-mediated antitumor responses depend on T and B cells. Normal or SCID BALB/c mice were injected with 2×10^5 MethA tumor cells. Treatment of established tumors was started 7 days later (except for untreated control mice in A). Mice were treated for 3 weeks with 50 μ g of B7.2-IgG twice a week (B and D) or with 5×10^5 irradiated MethA cells mixed with 50 μ g of B7.2-IgG followed by an additional dose of 50 μ g of B7.2-IgG 3 days later (C).

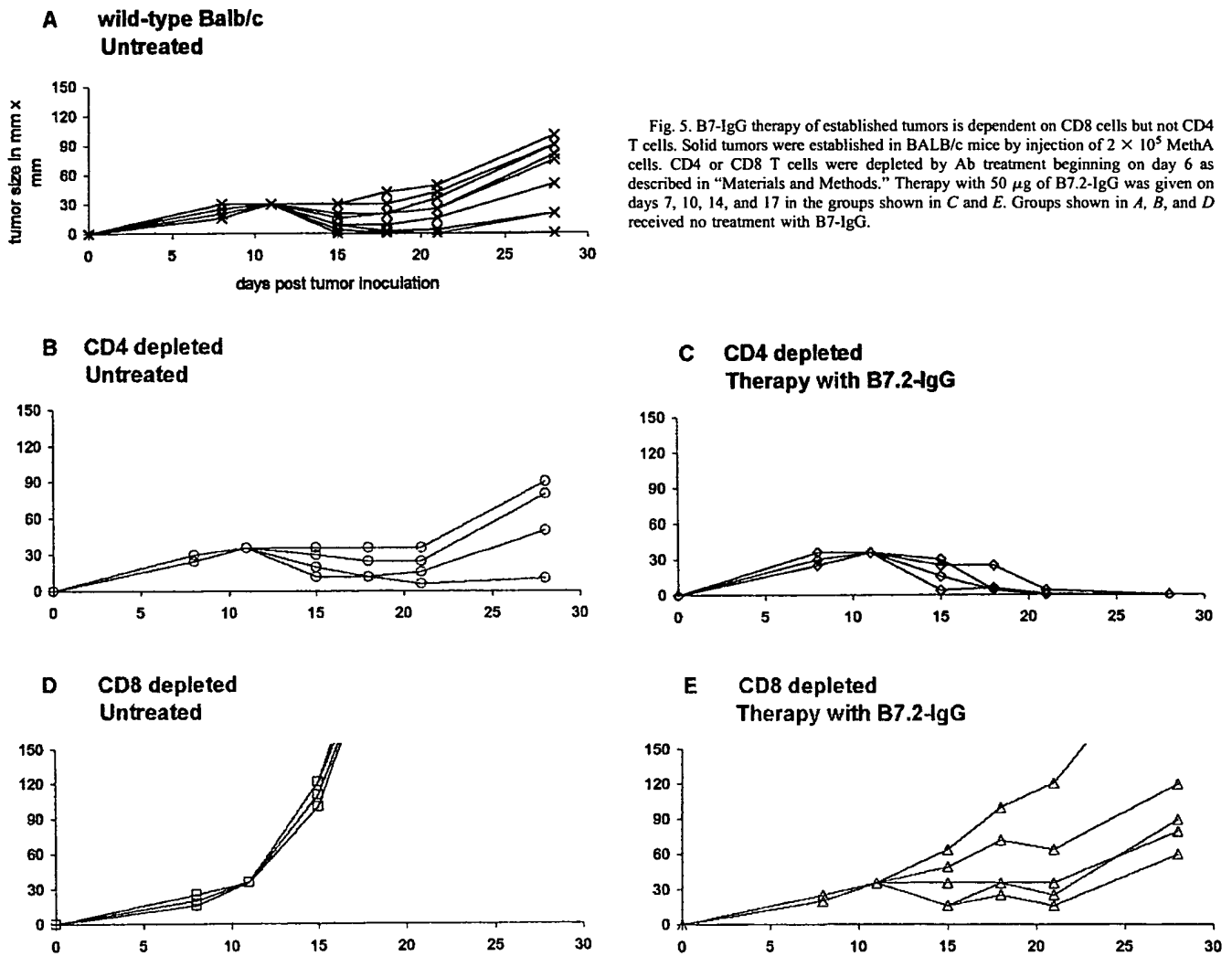


Fig. 5. B7-IgG therapy of established tumors is dependent on CD8 cells but not CD4 T cells. Solid tumors were established in BALB/c mice by injection of 2×10^5 MethA cells. CD4 or CD8 T cells were depleted by Ab treatment beginning on day 6 as described in "Materials and Methods." Therapy with 50 μ g of B7.2-IgG was given on days 7, 10, 14, and 17 in the groups shown in C and E. Groups shown in A, B, and D received no treatment with B7-IgG.

depends on tumor-specific immune responses is provided by rechalleng experiments. Mice that had been cured of established P815 or MethA tumors by therapy with B7.1-IgG or B7.2-IgG were rechallenged with the same tumors on the contralateral flank 60–110 days after the last vaccination. None of the rechallenged mice developed palpable tumors, regardless of whether they had been previously treated with B7.1-IgG or B7.2-IgG alone or with irradiated tumor cells mixed with either B7-IgG (data not shown). In total, these findings indicated that B7-IgG-enhanced antitumor responses were dependent on CD8 T cells and that memory responses were generated in mice cured by B7-IgG therapy.

B7-IgG-mediated Tumor Therapy Is IFN- γ Independent. Because IFN- γ plays an important role in antitumor immune surveillance and antitumor responses (40–42), we asked whether B7-IgG could cure established tumors independent of IFN- γ . Normal BALB/c or BALB/c-IFN- γ knockout mice bearing 7-day-old MethA tumors were treated with B7.2-IgG twice a week for 3 weeks or with irradiated tumor cells mixed with B7.2-IgG once a week for 3 weeks. Tumors grew more rapidly in the untreated IFN- γ -deficient mice compared with wild-type mice (Fig. 6, A and D). However, in both mouse strains, therapeutic treatment with B7.2-IgG alone (Fig. 6, B and E) or with irradiated tumor cells plus B7.2-IgG (Fig. 6, C and F) induced tumor regression and cure by about day 28. These results demonstrated that B7.2-IgG tumor therapy is IFN- γ independent.

DISCUSSION

We report here that fusion proteins linking B7.1 or B7.2 extracellular domains to the Fc region of murine IgG2a function as potent adjuvants for antitumor vaccines. B7.1-IgG or B7.2-IgG mixed with an irradiated tumor cell vaccine protected mice against a tumor challenge. More significantly, irradiated tumor cells mixed with B7.1-IgG or B7.2-IgG as an adjuvant were effective as therapeutic tumor vaccines in three strains of mice and in four tumor models. In all four tumor models, vaccination of tumor-bearing mice with irradiated tumor cells mixed with B7.1-IgG or B7.2-IgG led to regression of solid tumors and cure as measured by survival without detectable primary tumor for an observation period of 70–120 days. The potency of B7-IgG was best demonstrated in the poorly immunogenic B16/F10 melanoma model, where we achieved cure in up to 90% of the mice (Fig. 2). Most importantly, therapeutic treatment of tumor-bearing mice with B7-IgG alone also induced significant tumor regression and increased survival in all models tested. In fact, in three models (P815, MethA, and MB49) the results after treatment with B7-IgG alone were indistinguishable from treatments with irradiated tumor cell vaccines mixed with B7-IgG (Fig. 3). The B16/F10 tumor was the only model in which immunization with irradiated tumor mixed with either B7-IgG had a greater effect than treatment with B7-IgG alone (80% long-term survival *versus* 40% long-term survival, respectively). In this tumor model also, B7.2-IgG had a some-

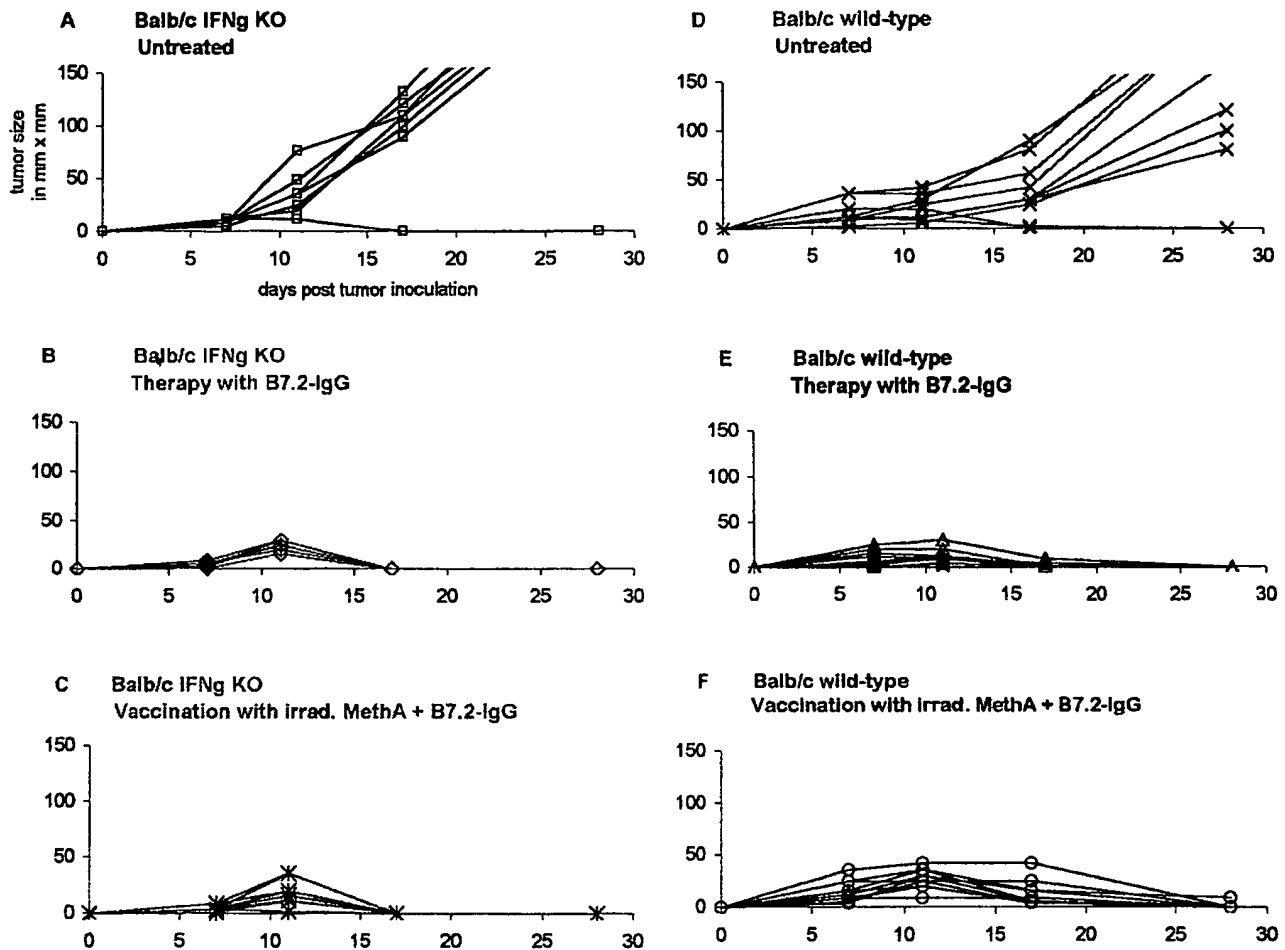


Fig. 6. B7-IgG therapy cures established MethA tumors in the absence of IFN- γ . MethA tumors were established in normal BALB/c mice (D and F) or IFN- γ knockout BALB/c mice (A-C) on day 0. Treatment with B7-IgG was started on day 7, with 50 μ g of B7.2-IgG given twice a week for 3 weeks (B and E). Groups shown in C and F were vaccinated with irradiated MethA tumor cells mixed with 50 μ g of B7.2-IgG on days 7, 14, and 21 and an additional dose of 50 μ g of B7.2-IgG on days 10, 14, and 24. Mice in the groups shown in A and D were left untreated.

what greater effect than B7.1-IgG. In summary, both B7.1-IgG and B7.2-IgG demonstrated strong antitumor activity in several established tumor models as therapeutic agents and as a vaccine adjuvant.

To determine whether the therapeutic antitumor activity of B7-IgG is immune-mediated, we tested B7.2-IgG in tumor-bearing mice lacking T and B cells, depleted of CD4 or CD8 cells, or lacking the capacity to generate IFN- γ . The absence of B7.2-IgG activity in SCID mice established the role of T and/or B cells (Fig. 4). Studies of mice depleted of CD4 or CD8 cells by Ab treatment revealed that CD8 cells are essential for the function of B7-IgG (Fig. 5). The role of CD4 cells may not be as clear. Although B7.2-IgG was active in mice depleted of CD4 cells, suggesting that CD4 cells are not essential for B7-IgG therapy, it is important to indicate that CD4 depletion was initiated 6 days after tumor inoculation. Thus, there may have been time to generate tumor-specific CD4 responses and to provide help in the initiation phase of the CD8 response. Depletion of CD4 T cells at the time of tumor inoculation may reveal a role for this T-cell subset. Nevertheless, our data show that CD8 cells, but not CD4 cells, need to be present during B7-IgG therapy. The rechallenge experiments provide further proof that the activity of B7-IgG is mediated through tumor-specific immune mechanisms and that memory responses are generated. It remains to be determined whether B7-IgG induces a new tumor-specific response or promotes the expansion and enhancement of existing yet ineffectual responses.

Perhaps the most remarkable finding is that the therapeutic effect of B7-IgG is independent of IFN- γ expression by the host. Therapeutic treatment with B7.2-IgG led to tumor cure in antitumor IFN- γ knockout mice (Fig. 6). The capacity of B7.2-IgG to enhance activity independent of IFN- γ is consistent with our observations in other studies that B7-IgG as a vaccine adjuvant enhances but does not shift type 1 and type 2 immune responses (30). This property of B7-IgG distinguishes it from other immune therapeutic approaches such as interleukin 2, IFN- γ , and interleukin 12, where IFN- γ plays a key role (43, 44). We are currently investigating other cytokines that may be responsible for the effects of B7-IgG. Granulocyte macrophage colony-stimulating factor is especially interesting because it has been shown to have potent antitumor activity when given as a vaccine adjuvant or transfected into tumor cells (45, 46). Moreover, cross-linking of CD28 has been shown to induce granulocyte macrophage colony-stimulating factor.

Several hypotheses can be proposed for the mechanism by which the B7-IgG fusion proteins assist the generation of novel antitumor responses or enhance existing antitumor responses. B7.1-IgG and B7.2-IgG can bind to both CD28 and CTLA-4 (as demonstrated by *in vitro* binding studies; data not shown). Therefore, B7-IgG has the potential to enhance costimulatory effects through CD28 as well as to prevent negative signals triggered through CTLA-4. The role and effect of B7-IgG may shift, depending on the condition of the T-cell

response. For naïve T cells, which initially do not express CTLA-4, costimulation through CD28 is likely to be most important. CD28 signaling may be increased by aggregation of B7-IgG, mediated through Fc binding on APCs. This is consistent with our observation that in prophylactic models, B7-IgG requires the Fc binding function (Table 1). In contrast, in therapeutic settings, the stimulation of activated or memory T cells recognizing tumor antigens may require less costimulation through CD28 (47). Thus, in therapeutic settings, aggregation of B7-IgG may be less important, which is consistent with our finding that in this circumstance, wild-type and Fc-mutated forms of B7-IgG are equally effective (data not shown). In addition, in therapeutic settings, soluble B7-IgG may bind with high affinity to CTLA-4 on activated T cells to block its negative signal, thereby enhancing the activity of tumor-specific T cells or preventing their down-regulation. A better characterization of the function of B7-IgG remains the focus of future studies and will help us to understand the differences in various tumor therapy approaches targeting the B7-CD28/CTLA-4 costimulatory pathway.

Previous reports described the successful therapeutic treatment of established murine tumors with Ab blocking CTLA-4 (27). This approach induced the regression of strongly immunogenic tumors but not poorly immunogenic tumors (40), in contrast to our findings that B7.1-IgG and B7.2-IgG were effective as therapeutic agents or vaccine adjuvant even in the poorly immunogenic tumors MB49 and B16/F10. These observations suggest that B7-IgG may work through different mechanisms than anti-CTLA-4 Ab, has greater potency in blocking CTLA-4, or has a dual function in binding to CD28 and CTLA-4. Another approach targeting the costimulation pathway in tumor therapy focuses on transfection or transduction of B7 into tumor cells. However, this approach has only been successful in prophylactic tumor models (17–25). Little success using B7-transfected tumor cells has been achieved in therapeutic tumor models, as corroborated by our findings with the B7.1 transfectants of P815 and B16/F10 (Table 1; data not shown). The relative ineffectiveness of tumor cells transfected with B7 as compared with injection of soluble protein may reflect quantitative differences in the number of available B7 molecules. However, the difference may also be explained by the dual function of B7 molecules. Membrane-bound B7 expressed by APCs can cross-link CD28 and costimulate T cells, but it can also cross-link CTLA-4 on activated T cells and trigger a negative signal. B7 expressed on transfected tumor cells may have comparable functions. In contrast, soluble B7-IgG may block rather than trigger signaling through CTLA-4, sustaining the activation of tumor-specific T cells. Studies to better understand the mechanism of the immune-enhancing effect of B7-IgG are in progress.

Recently, Moro *et al.* (48) reported the therapeutic antitumor efficacy of B7-immunoglobulin fusion proteins. Their approach is comparable with vaccination with B7-transfected tumor cells because they indirectly targeted B7-immunoglobulin to the tumor with Ab recognizing tumor-specific antigens. In contrast to our findings, they did not observe antitumor activity when B7-immunoglobulin was administered in soluble form without the targeting Ab. Without having compared the different fusion proteins, we could speculate that the differences in production and the tagging with biotin may affect the pharmacokinetics of the molecules. In preliminary studies, we have determined long half-lives of 80–90 h in mice for our B7-IgG proteins. It is worth noting that despite the extended systemic exposure with these costimulatory molecules, we have not observed any morbidity or signs of autoimmune diseases in mice kept for more than 4 months after therapy.

In summary, B7-IgG fusion proteins appear to be effective antitumor agents that seem to be safe and can readily be administered in the clinic and manufactured. Their potency in stimulating immune re-

sponses and cure in multiple murine tumor models suggests clinical potential as an adjuvant and therapy for oncology and for other clinical indications.

ACKNOWLEDGMENTS

We thank Kathy Tomkinson for generation of the B7-IgG-expressing CHO cell lines, the MBGE group for providing peptides, the Antibody Technology Group for providing purified monoclonal Abs, and Ronald Li for the pharmacokinetic analysis. We also thank Dr. Thomas Gajewski for helpful discussion during the course of this work and for reviewing the manuscript.

REFERENCES

- Allison, J. P., and Krummel, M. F. The yin and yang of T cell costimulation. *Science* (Washington DC), 270: 932–933, 1995.
- Guinan, E. C., Gribben, J. G., Boussiotis, V. A., Freeman, G. J., and Nadler, L. M. Pivotal role of the B7:CD28 pathway in transplantation tolerance and tumor immunity. *Blood*, 84: 3261–3282, 1995.
- Bluestone, J. A., and Lechler, R. I. Transplantation. *Curr. Opin. Immunol.*, 7: 617–619, 1995.
- Lenschow, D. J., Herold, K. C., Rhee, L., Patel, B., Koons, A., Qin, H. Y., Fuchs, E., Singh, B., Thompson, C. B., and Bluestone, J. A. CD28/B7 regulation of Th1 and Th2 subsets in the development of autoimmune diabetes. *Immunity*, 5: 285–293, 1996.
- Bluestone, J. A. Is CTLA-4 a master switch for peripheral T cell tolerance? *J. Immunol.*, 158: 1989–1993, 1997.
- Allison, J. P., Hurwitz, A. A., and Leach, D. R. Manipulation of costimulatory signals to enhance antitumor T-cell responses. *Curr. Opin. Immunol.*, 7: 682–686, 1995.
- Boon, T., Cerottini, J. C., van den Eynde, B., van der Bruggen, P., and van Pel, A. Tumor antigens recognized by T lymphocytes. *Annu. Rev. Immunol.*, 12: 337–365, 1994.
- Pardoll, D. M. Cancer vaccines. *Nat. Med.*, 4: 525–531, 1998.
- Biddison, W. E., and Palmer, J. C. Development of tumor cell resistance to syngeneic cell-mediated cytotoxicity during growth of ascitic mastocytoma P815Y. *Proc. Natl. Acad. Sci. USA*, 74: 329–333, 1977.
- Lehmann, F., Marchand, M., Hainaut, P., Pouillart, P., Sastre, X., Ikeda, H., Boon, T., and Coulic, P. G. Differences in the antigens recognized by cytolytic T cells on two successive metastases of a melanoma patient are consistent with immune selection. *Eur. J. Immunol.*, 25: 340–347, 1995.
- Momburg, F., and Koch, S. Selective loss of symbol 98₂-microglobulin mRNA in human colon carcinoma. *J. Exp. Med.*, 169: 309–314, 1989.
- Rivoltini, L., Barracchini, K. C., Viggiano, V., Kawakami, Y., Smith, A., Mixon, A., Restifo, N. P., Topalian, S. L., Simonis, T. B., and Rosenberg, S. A. Quantitative correlation between HLA class I allele expression and recognition of melanoma cells by antigen-specific cytotoxic T lymphocytes. *Cancer Res.*, 55: 3149–3157, 1995.
- Uytendoven, C., Maryanski, J., and Boon, T. Escape of mouse mastocytoma P815 after nearly complete rejection is due to antigen-loss variants rather than immunosuppression. *J. Exp. Med.*, 157: 1040–1052, 1983.
- Chamberlain, R. S., Carroll, M. W., Bronte, V., Hwu, P., Warren, S., Yang, J. C., Nishimura, M., Moss, B., Rosenberg, S. A., and Restifo, N. P. Costimulation enhances the active immunotherapy effect of recombinant anticancer vaccines. *Cancer Res.*, 56: 2832–2836, 1996.
- Ding, L., and Shevach, E. M. Activation of CD4⁺ T cells by delivery of the B7 costimulatory signal on bystander antigen-presenting cells (*trans*-costimulation). *Eur. J. Immunol.*, 24: 859–866, 1994.
- Gimmi, C. D., Freeman, G., Gribben, J. G., Gray, G., and Nadler, L. M. Human T cell clonal anergy is induced by antigen presentation in the absence of B7 costimulation. *Proc. Natl. Acad. Sci. USA*, 90: 6586–6590, 1993.
- Yang, G., Mizuno, M. T., Hellstrom, K. E., and Chen, L. B7-negative *versus* B7-positive P815 tumor: differential requirements for priming of an antitumor immune response in lymph nodes. *J. Immunol.*, 158: 851–858, 1997.
- Cavallo, F., Martin-Fontecha, A., Bellone, M., Heltai, S., Gatti, E., Freshci, M., Forni, G., Dellabona, P., and Casorati, G. Co-expression of B7-1 and ICAM-1 on tumors is required for rejection and the establishment of a memory response. *Eur. J. Immunol.*, 25: 1154–1162, 1995.
- Chen, L., Ashe, S., Brady, W. A., Hellstrom, I., Hellstrom, K. E., Ledbetter, J. A., McGowan, P., and Linsley, P. S. Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell*, 71: 1093–1102, 1992.
- Chen, L., McGowan, P., Ashe, S., Johnston, J., Li, Y., Hellstrom, I., and Hellstrom, K. E. Tumor immunogenicity determines the effect of B7 costimulation on T cell-mediated tumor immunity. *J. Exp. Med.*, 179: 523–532, 1994.
- Dunussi-Joannopoulos, K., Weinstein, H. J., Nickerson, P. W., Strom, T. B., Burakoff, S. J., Croop, J. M., and Arceci, R. J. Irradiated B7-1 transduced primary acute myelogenous leukemia (AML) cells can be used as therapeutic vaccines in murine AML. *Blood*, 87: 2938–2946, 1996.
- Li, Y., McGowan, P., Hellstrom, I., Hellstrom, K. E., and Chen, L. Costimulation of tumor-reactive CD4⁺ and CD8⁺ T lymphocytes by B7, a natural ligand for CD28, can be used to treat established mouse melanoma. *J. Immunol.*, 153: 421–428, 1994.
- Townsend, S. E., and Allison, J. P. Tumor rejection after direct costimulation of CD8⁺ T cells by B7-transfected melanoma cells. *Science* (Washington DC), 259: 368–370, 1993.

24. Yang, G., Hellstrom, K. E., Hellstrom, I., and Chen, L. Antitumor immunity elicited by tumor cells transfected with B7-2, a second ligand for CD28/CTLA-4 costimulatory molecules. *J. Immunol.*, 154: 2794–2800, 1995.
25. La Motte, R. N., Rubin, M. A., Barr, E., Leiden, J. M., Bluestone, J. A., and Mokr, M. B. Therapeutic effectiveness of the immunity elicited by P815 tumor cells engineered to express the B7-2 costimulatory molecule. *Cancer Immunol. Immunother.*, 42: 161–169, 1996.
26. Putzer, B. M., Hiitt, M., Muller, W. J., Emtage, P., Gaudie, J., and Graham, F. L. Interleukin 12 and B7-1 costimulatory molecule expressed by an adenovirus vector act synergistically to facilitate tumor regression. *Proc. Natl. Acad. Sci. USA*, 94: 10889–10994, 1997.
27. Leach, D. R., Krummel, M. F., and Allison, J. P. Enhancement of antitumor immunity by CTLA-4 blockade. *Science* (Washington DC), 271: 1734–1736, 1996.
28. Zheng, P., Wu, Y., Guo, Y., Lee, C., and Liu, Y. B7-CTLA4 interaction enhances both production of antitumor cytotoxic T lymphocytes and resistance to tumor challenge. *Proc. Natl. Acad. Sci. USA*, 95: 6284–6289, 1998.
29. Fields, P. E., Finch, R. J., Gray, G. S., Zollner, R., Thomas, J. L., Sturmhoefel, K., Lee, K., Wolf, S., Gajewski, T. F., and Fitch, F. W. B7.1 is a quantitatively stronger costimulus than B7.2 in the activation of naive CD8⁺ TCR-transgenic T cells. *J. Immunol.*, 161: 5268–5275, 1998.
30. Swiniarski, H. M., Sturmhoefel, K., Lee, K., Gray, G. S., Thomas, J. L., Wolf, S. F., Dorner, A. J., and O'Toole, M. Immune response enhancement by *in vivo* administration of B7.2-Ig, a soluble costimulatory protein. *Clin. Immunol.*, in press, 1999.
31. Freeman, G., Gray, G., Gimmi, C., Lombard, D., Zhou, L., White, M., Fingerhuth, J., Gribben, J., and Nadler, L. Structure, expression, and T cell costimulatory activity of the murine homologue of the human B lymphocyte activation antigen B7. *J. Exp. Med.*, 174: 625–631, 1991.
32. Freeman, G., Borriello, F., Hodes, R., Reiser, H., Gribben, J., Ng, J., Kim, J., Goldberg, J., Hathcock, K., Laszlo, G., *et al.* Murine B7-2, an alternative CTLA4 counter-receptor that costimulates T cell proliferation and interleukin 2 production. *J. Exp. Med.*, 178: 2185–2192, 1993.
33. Steurer, W., Nickerson, P., Steele, A., Steiger, J., Zheng, X., and Strom, T. *Ex vivo* coating of islet cell allografts with murine CTLA4/Fc promotes graft tolerance. *J. Immunol.*, 155: 1165–1174, 1995.
34. Kaufman, R. J., Davies, M. V., Wasley, L. C., and Michnick, D. Improved vectors for stable expression of foreign genes in mammalian cells by use of the untranslated leader sequence from EMC virus. *Nucleic Acids Res.*, 19: 4485–4490, 1991.
35. DeLeo, A., Shiku, H., Takahashi, T., John, M., and Old, L. Cell surface antigens of chemically induced sarcomas of the mouse. I. Murine leukemia virus-related antigens and alloantigens on cultured fibroblasts and sarcoma cells: description of a unique antigen on BALB/c Meth A sarcoma. *J. Exp. Med.*, 146: 720–734, 1977.
36. Poste, G., Doll, J., Hart, I., and Fidler, I. *In vitro* selection of murine B16 melanoma variants with enhanced tissue-invasive properties. *Cancer Res.*, 40: 1636–1644, 1980.
37. Hunter, S. E., Waldburger, K. E., Thibodeaux, D. K., Schaub, R. G., Goldman, S. J., and Leonard, J. P. Immunoregulation by interleukin-12 in MB49.1 tumor-bearing mice: cellular and cytokine-mediated effector mechanisms. *Eur. J. Immunol.*, 27: 3438–3446, 1997.
38. Gajewski, T. F., Fallarino, F., Uyttenhove, C., and Boon, T. Tumor rejection requires a CTLA4 ligand provided by the host or expressed on the tumor: superiority of B7-1 over B7-2 for active tumor immunization. *J. Immunol.*, 156: 2909–2917, 1996.
39. Uyttenhove, C., van Snick, J., and Boon, T. Immunogenic variants obtained by mutagenesis of mouse mastocytoma P815. I. Rejection by syngeneic mice. *J. Exp. Med.*, 152: 1175–1183, 1980.
40. Hurwitz, A. A., Townsend, S. E., Yu, T. F., Wallin, J. A., and Allison, J. P. Enhancement of the anti-tumor immune response using a combination of interferon-Symbol" § 16 and B7 expression in an experimental mammary carcinoma. *Int. J. Cancer*, 77: 107–113, 1998.
41. Kaplan, D. H., Shankaran, V., Dighe, A. S., Stockert, E., Aguet, M., Old, L. J., and Schreiber, R. D. Demonstration of an interferon γ 16-dependent tumor surveillance system in immunocompetent mice. *Proc. Natl. Acad. Sci. USA*, 95: 7556–7561, 1998.
42. Musiani, P., Modesti, A., Giovarelli, M., Cavallo, F., Colombo, M. P., Lollini, P.-L., and Forni, G. Cytokines, tumor-cell death and immunogenicity: a question of choice. *Immunol. Today*, 18: 31–36, 1997.
43. Coughlin, C. M., Salhany, K. E., Gee, M. S., LaTemple, D. C., Kotenko, S., Ma, X.-J., Gri, G., Wysocka, M., Kim, J. E., Liu, L., Liao, F., Farber, J. M., Pestka, S., Trinchieri, G., and Lee, W. M. F. Tumor cell responses to IFN- γ affect tumorigenicity and response to IL-12 therapy and antiangiogenesis. *Immunity*, 9: 25–34, 1998.
44. Gately, M. K., Warrier, R. R., Honasoge, S., Carvajal, D. M., Faherty, D. A., Connaughton, S. E., Anderson, T. D., Sarmiento, U., Hubbard, B. R., and Murphy, M. Administration of recombinant IL-12 to normal mice enhances cytolytic lymphocyte activity and induces production of IFN- γ *in vivo*. *Int. Immunol.*, 6: 157–167, 1994.
45. Dranoff, G., Jaffee, E., Lazenby, A., Golumbek, P., Levitsky, H., Brose, K., Jackson, V., Hamada, H., Pardoll, D., and Mulligan, R. C. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc. Natl. Acad. Sci. USA*, 90: 3539–3543, 1993.
46. Kruger, M., Van Gool, S., Peng, X. H., Coorevits, L., Casteels-Van, D. M., and Ceuppens, J. L. Production of granulocyte-macrophage colony-stimulating factor by T cells is regulated by B7 and IL-1symbol 98. *Immunology*, 88: 49–54, 1996.
47. Kuiper, H., Brouwer, M., de Boer, M., Parren, P., and van Lier, R. A. Differences in responsiveness to CD3 stimulation between naive and memory CD4⁺ T cells cannot be overcome by CD28 costimulation. *Eur. J. Immunol.*, 24: 1956–1960, 1994.
48. Moro, M., Gasparri, A. M., Pagano, S., Bellone, M., Tornaghi, P., Veglia, F., Corti, A., Casorati, G., and Dellabona, P. Induction of therapeutic T-cell immunity by tumor targeting with soluble recombinant B7-immunoglobulin costimulatory molecules. *Cancer Res.*, 59: 2650–2656, 1999.



Induction of Therapeutic T-Cell Immunity by Tumor Targeting with Soluble Recombinant B7-Immunoglobulin Costimulatory Molecules¹

Monica Moro,² Anna Maria Gasparri, Stefano Pagano, Matteo Bellone, Paola Tornaghi, Fabrizio Veglia, Angelo Corti, Giulia Casorati,³ and Paolo Dellabona³

Immunochimistry Unit [M. M., S. P., P. T., G. C., P. D.], Immunobiotechnology Unit, DIBIT [A. M. G., A. C.], Tumor Immunology Laboratory [M. B.], Biostatistic Unit [F. V.], and Cancer Immunotherapy and Gene Therapy Program [M. M., A. M. G., S. P., M. B., P. T., A. C., G. C., P. D.], H. San Raffaele Scientific Institute, I-20132, Milan, Italy

ABSTRACT

Tumor targeting with immunomodulatory molecules is an attractive strategy to enhance the host's antitumor response. Expression of CD80 (B7-1) and CD86 (B7-2) costimulatory molecules in tumor cells has proven to be an efficient way to enhance their immunogenicity. Here, we studied the effects of tumor targeting with biotinylated recombinant soluble B7-1 and B7-2 immunoglobulin G molecules (bio-B7-IgG) using a pretargeting approach based on the sequential use of a biotinylated antitumor monoclonal antibody and avidin. Mouse RMA T-lymphoma cells bearing either bio-B7-1-IgG or bio-B7-2-IgG on their surface prime *in vitro* naive CD8⁺ CTLs, which are highly effective in adoptive immunotherapy, and induce therapeutic immunity when injected in tumor-bearing animals. *In vivo* targeting of established RMA tumors with bio-B7-IgG either cures tumor-bearing mice or significantly prolongs their survival. The antitumor response induced by targeted bio-B7-IgG depends on both CD4⁺ and CD8⁺ T cells. Moreover, tumor targeting with bio-B7-IgG *in vivo* is critical for both expansion in lymphoid organs and mobilization into the tumor of tumor-specific CD8⁺ CTLs. When targeting is performed on poorly immunogenic TS/A mammary adenocarcinoma, only bio-B7-1-IgG primes naive CTLs *in vitro* and cures or significantly prolongs the survival of tumor-bearing mice *in vivo*, confirming that the two costimulatory molecules are not redundant with this tumor. Altogether, these data suggest that tumor avidination and targeting with soluble bio-B7-IgG may represent a promising strategy to enhance the antitumor response in the host.

INTRODUCTION

Tumor cells are antigenic and can be recognized by autologous tumor-specific T cells (1). These findings provide a strong rationale for the application of immunotherapy to cancer, aimed at activating the potential antitumor T-cell repertoire present in each patient.

For optimal induction, T cells require two activating signals (2). Signal one is generated by the interaction of the TCRs⁴ with their cognate peptide-MHC complex. Signal two is induced through the binding of particular T-cell-activating molecules with their counter-receptors, called costimulatory molecules. The best characterized costimulatory pairs are the B7-1 and B7-2 molecules, which are expressed mainly by professional antigen-presenting cells, and their T-cell counterreceptors CD28 and CTLA4 (2-4). The lack of B7 expression by tumor cells may partially explain why they often escape the host's immune system (2, 5). Indeed, experimental tumors, which are sufficiently antigenic, become immunogenic when transfected with B7-1 or B7-2 genes and are rejected by syngeneic animals

(6-10). The main effect of the expression of B7 on tumor cells is the activation of a specific T-cell response (6-10). Moreover, it has also been shown that the expression of B7 on tumors activates the innate arm of the immune system, which plays a major role in the rejection of some tumors (11-14). Most of these data have been generated with tumors transduced with the genes coding for B7-1 or B7-2. We have devised an alternative approach that can more easily generate tumor cells bearing the extracellular domain of B7 on their surface. Moreover, this approach can target the expression of B7 costimulatory molecules on tumor cells *in vivo*, without requiring the transduction of tumor cells with the genes encoding the costimulatory molecules. This approach is based on the sequential incubation of cultured tumor cells with biotinylated antibodies specific for a membrane TAA, avidin, and biotinylated soluble B7-1 or B7-2 fused with the IgG constant regions (bio-B7-IgG). We show that targeting mouse tumor cells with bio-B7-IgG *in vitro* is an efficient way to expand tumor-specific CTLs for adoptive immunotherapy and to generate therapeutic nonreplicating whole cell vaccines. More importantly, *in vivo* treatment of tumor-bearing animals with bio-B7-IgG induces a therapeutic T-cell immunity.

MATERIALS AND METHODS

Mice. BALB/c and C57BL/6 4-8-week-old female mice were purchased from Charles River Laboratories (Calco, Italy).

Tumor Cell Lines. The Rauscher virus-induced RMA T-lymphoma of C57BL/6 origin (H-2^b) and the spontaneously arisen, moderately differentiated TS/A mammary adenocarcinoma of BALB/c origin (H-2^d) were maintained *in vitro* in complete medium (RPMI 1640 with 5% FCS, 100 units/ml penicillin, 100 units/ml streptomycin, and 2.5×10^{-5} M 2-mercaptoethanol). RMA cells express the endogenous Thy 1.2 antigen, whereas TS/A cells do not.

Thy 1.1 Cloning and Transfection. Both the cloning of mouse Thy 1.1 cDNA and the generation of RMA cells expressing the Thy 1.1 antigen on their surface (RMA-T cells) have been described previously (15). TS/A cells expressing the Thy 1.1 antigen (TS/A-T cells) were obtained following the same protocols.

Generation of Recombinant Soluble B7-Ig. The ectodomain of human B7-1 was obtained from full-length cDNA (11) by PCR using the following oligonucleotides: (a) 5'-CCTGAGCTCCTGAAGCCATGGGCCACACACGG-3', 5' primer; and (b) 5'-TCTGACTTACCATCAGGAAAAATGCTCTTGCTT-3, 3' primer. The extracellular region of human B7-2 was cloned from full-length cDNA (10) by PCR using the following oligonucleotides: (a) 5'-GATATGAGCTCACAGCAGAAGCAG-3', 5' primer; and (b) 5'-ACTTACCTGAGCTCTGGGGGAGG-3', 3' primer. B7-1 and B7-2 cDNAs were cloned into the mammalian expression vectors pCD4-Hy1 and pCD4-Hy3, respectively (Ref. 16; kindly obtained from Dr. K. Karjalainen, Basel Institute for Immunology, Basel, Switzerland), replacing the human CD4 ectodomain sequence. The resulting plasmids (p-hB7-1-IgG and p-hB7-2-IgG) were used to transfect mouse J558L plasmacytoma cells by protoplast fusion (16). Transfected cells were selected by growing them in culture medium containing 5 μ g/ml mycophenolic acid and 100 μ g/ml xanthine and screening them for the secretion of B7-IgG soluble chimeric proteins by ELISA. Positive clones were subcloned and expanded to produce the soluble protein. The B7-IgG molecules were purified from culture supernatants by protein G-Sepharose affinity chromatography.

Preparation of bio-19E12 mAb and bio-B7-IgG. Biotinylation of the anti-Thy 1.1 mAb 19E12 was performed as described previously (15). To

Received 12/7/98; accepted 4/2/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Supported by grants from the Associazione Italiana per la Ricerca sul Cancro and Boehringer Mannheim.

² M. M. was supported by a fellowship from the Associazione Italiana per la Ricerca sul Cancro.

³ To whom requests for reprints should be addressed, at Unità d'Immunochimica, DIBIT, Istituto Scientifico San Raffaele, Via Olgettina 58, Milan 20132, Italy. Phone: 39-2-2643-4727; Fax: 39-2-2643-4723; E-mail: dellabona.paolo@hsr.it.

⁴ The abbreviations used are: TCR, T-cell receptor; TAA, tumor-associated antigens; NAV, neutravidin; NWSC, nylon wool spleen cells; Ig, immunoglobulin; mAb, monoclonal antibody; IL, interleukin; hIL, human recombinant IL.

produce 19E12 F(ab')₂ fragments, 2 mg of mAb were mixed with 5 mg of pepsin in 6 ml of 0.1 M sodium citrate (pH 4.5) and incubated for 2 h at 37°C. After dialysis against 1.5 M glycine and 3 M sodium chloride, the Fab fragments were separated from Fc-containing molecules by protein A-Sepharose affinity chromatography. The final product consisted of 90% of F(ab')₂ fragments with minor contamination by Fab fragments, as judged from SDS-PAGE analysis.

Biotinylation of B7-Ig was achieved by mixing 0.5 mg of the recombinant molecule at 1 mg/ml of 0.1 M sodium bicarbonate (pH 8.5) with 6 mg of sulfo-NSH-LC-biotin (Pierce Chemical Co., Rockford, IL) at 1 mg/ml in water. After 30 min at 25°C, the reaction was stopped by dialyzing against PBS. The endotoxin content of all of the biotinylated molecules used for this study was determined by the *Limulus* amebocyte lysate Pyrogen (BioWhittaker, Walkersville, MD) to be ≤ 0.06 EU/ml.

In Vitro Targeting of Tumor Cells with bio-B7-IgG. RMA-T or TS/A-T cells (1×10^6 cells/ml) were mixed with 1 μ g of bio-19E12 mAb in 50 μ l of PBS containing 2% FCS (PBS/FCS) and incubated for 10 min on ice. After washing, the cells were incubated for 10 min on ice with 1 μ g of NAV (a deglycosylated avidin with a neutral isoelectric point; Pierce Chemical Co.) in 50 μ l of PBS/FCS, washed again, and finally resuspended in 50 μ l of PBS/FCS containing 3 μ g of bio-B7-Ig. After 10 min on ice, the cells were washed and used for the subsequent experiments.

In Vitro Priming of Tumor-specific CTLs. *In vitro* priming of tumor-specific CTLs was performed according to our published protocol (11). CD8⁺ T cells (80–90% pure) were further purified from NWSCs by complement-mediated lysis of CD4⁺ T cells using the anti-CD4 mAb GK1.5 and rabbit complement (LowTox Cedarline). Nonreplicating tumor cells were generated by incubating 10^7 cells with 100 μ g of mitomycin C in 1 ml of medium for 45 min at 37°C. CTLs were generated by culturing 10 – 20×10^6 CD8⁺ T cells with 2 – 4×10^6 nonreplicating RMA-T cells bearing bio-B7-IgG or bio-IgG in a 6-well plate containing 5 ml of complete medium. After 48 h of culture, hrIL-2 was added at 10 units/ml (Hofman-LaRoche, Basel, Switzerland). When using nonreplicating TS/A-T cells bearing bio-B7-IgG or bio-IgG to prime naive CTLs, total spleen cells were used. After 48 h, hrIL-2 and hrIL-7 (Boehringer Mannheim, Penzberg, Germany) were added at 10 units/ml each. In the inhibition experiments, either purified anti-CD8 mAb 53.6.72, human CTLA-4 IgG1, anti-MHC class I K^b mAb Y3, or anti-D^b mAb B22.249 was added at the beginning of the cocultures at a final concentration of 1 μ g/ml. Long-term antitumor CTL lines were maintained by restimulating T cells every 7–10 days with nonreplicating RMA-T cells prepulsed with bio-B7-IgG in the presence of 20 units/ml hrIL-2. To generate an alloreactive CTL line, 10 – 20×10^6 NWSCs from a naive C57BL/6 mouse were cultured with 2 – 4×10^6 nonreplicating J558L plasmacytoma cells of BALB/c origin and expanded as described above.

Cytotoxicity Assays. Blasts at day 6 of culture were tested in a standard 4-h ⁵¹Cr release assay (11). ⁵¹Cr spontaneous release was always <25% of the maximal (1 N HCl) ⁵¹Cr release. The percentage of specific ⁵¹Cr release was calculated as follows: (experimental cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm) $\times 100$.

Cytofluorimetric Studies. The presence of bio-B7-Ig on tumor cells after *in vitro* or *in vivo* targeting (see below) was assessed by cell staining with FITC-conjugated goat antihuman IgG antiserum (Southern Biotech., Birmingham, AL). The phenotype of spleen cells was determined by tricolor cytofluorimetric analysis using FITC-labeled rat antimouse CD8 α mAb, R-phycoerythrin-labeled rat antimouse CD69L mAb or R-phycoerythrin-labeled rat antimouse CD44 mAb, and biotinylated mouse antimouse V β 5.1–5.2 TCR mAb, followed by Cychrome-conjugated streptavidin (all reagents were from Pharmingen). Analysis was performed using a FACScan cytofluorimeter (Becton Dickinson, Palo Alto, CA).

In Vivo Studies. All *in vivo* studies were approved by the Ethical Committee of the Istituto Scientifico San Raffaele and performed according to its guidelines. To evaluate the therapeutic efficacy of nonreplicating RMA-T cells bearing bio-B7-IgG, 3×10^4 living parental RMA cells were injected s.c. into the left flank of B6 mice. Three days later, these mice began the therapeutic regimen, which consisted of six s.c. contralateral injections of 1×10^6 mitomycin C-treated RMA-T cells bearing bio-B7-IgG, administered twice a week for 3 weeks.

In adoptive transfer experiments, 3×10^4 living parental RMA cells were

injected s.c. into mice. After 72 h, 10^6 anti-RMA CTLs were injected i.p. into mice, together with hrIL-2 (2000 units) given once a day for the next 3 days.

For *in vivo* targeting experiments, mice bearing either a 24-h or a 72-h-old RMA-T tumor generated by the s.c. injection of 3×10^4 living cells received 50 μ g of bio-19E12 mAb F(ab')₂ fragments i.p., followed 24 h later by NAV (30 μ g; i.p.) and 6 h later by bio-B7-IgG or bio-IgG (50 μ g; i.p.). For the TS/A-T tumor model, 4×10^4 living cells were injected s.c., followed 48 h later by the i.p. injection of 50 μ g of bio-19E12 mAb (whole mAb). NAV and bio-B7-IgG molecules were then injected i.p. 24 h and 6 h, respectively, after the anti-Thy 1.1 mAb.

For *in vivo* depletion experiments, 300 μ g of either purified rat IgG, anti-CD4 GK1.5, or anti-CD8 53-6-72 mAb were injected i.p. into mice 48 h and 24 h before the tumor inoculum. This regimen causes the depletion of more than 90% of target lymphocytes from both the spleen and lymph nodes. An injection of the same amount of antibody was given once a week until the completion of the experiment.

To assess the development of a memory response, 25–30 days after the primary inoculum, tumor-free mice were challenged contralaterally with 10^5 RMA or TS/A parental cells and scored for tumor growth.

Immunohistochemistry Analysis of Tumor Sections. Animals bearing a 24-h-old RMA-T tumor underwent the three-step targeting protocol. After 13 days, tumors were excised, fixed with 4% paraformaldehyde, embedded in OCT (Miles, Inc., Elkhart, IN), and frozen in liquid nitrogen. Cryostat sections (10 μ m) were stained with FITC-labeled anti-CD4 and anti-CD8 mAb (PharMingen).

Statistical Analysis. All experiments performed *in vivo* were repeated at least twice with groups of 5–10 mice, giving homogeneous results. Survival curve data were accumulated and analyzed using the log-rank statistic test.

RESULTS

Tumor Cells Bearing bio-B7-IgG Prime Specific CTLs *In Vitro*.

The efficacy of the costimulatory signal provided by targeted bio-B7-IgG molecules was first evaluated *in vitro* using RMA lymphoma cells bearing soluble B7-IgG molecules to prime tumor-specific naive CTLs. This tumor was chosen because it induces antitumor T cells when transfected with either full-length B7-1 or B7-2 cDNA (10). To enable specific binding of B7-IgG to tumor cells, we exploited a three-step-dependent strategy based on sequential incubations with: (a) biotin-conjugated mAb specific for a membrane TAA; (b) avidin; and (c) biotin-conjugated B7-IgG. To generate a surface TAA selectively expressed by tumor cells, RMA cells were transfected with the cDNA encoding the Thy 1.1 allele, which is not expressed by C57BL/6 mice. Thy 1.1 is poorly immunogenic in C57BL/6 mice and does not alter the growth rate of transfected tumor cells either *in vitro* or *in vivo* (data not shown). Bio-B7-IgG molecules bound to RMA-T cells with the three-step approach were stable on the surface of the tumor cells for at least 24 h at 37°C, indicating that the complexes are not internalized or are poorly internalized (data not shown). Moreover, we determined that both bio-B7-1-IgG and bio-B7-2-IgG were functional because they bind Chinese hamster ovary cells expressing transfected CTLA-4 on the surface and costimulate IL-2 production in a mouse T-cell hybridoma activated by a suboptimal concentration of anti-CD3 mAb (data not shown).

The results of coculture experiments with NWSCs from naive animals showed that RMA-T cells coated with either bio-B7-1-IgG or bio-B7-2-IgG indeed prime tumor-specific CTLs *in vitro* (Fig. 1A). The addition of either soluble CTLA-4-Ig, anti-CD8 mAb, or anti-D^b mAb, but not rat IgG, during the coculture abolished the priming (Fig. 1B). Thus, the B7 ectodomain of the conjugate and the D^b-peptide complexes on RMA as well as the expression of CD8 on CTLs were all necessary for the priming.

Adoptive Immunotherapy of Tumor-bearing Mice with CTLs Primed *In Vitro* by RMA-T Cells Coated with bio-B7-IgG. We next assessed whether RMA-T cells coated with bio-B7-IgG could

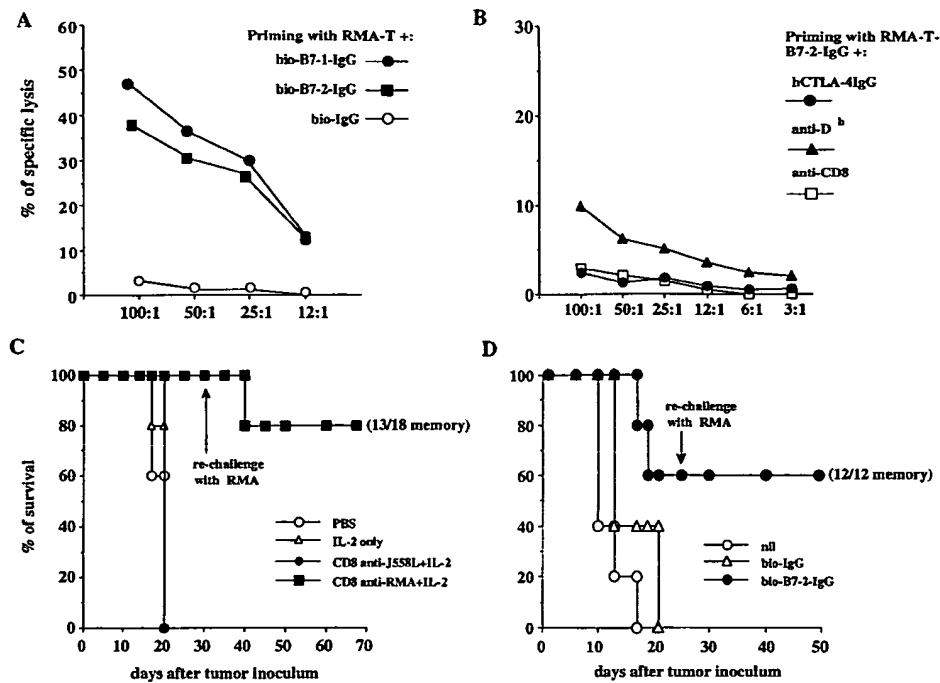


Fig. 1. Antitumor responses induced by RMA tumor cells targeted with bio-B7-IgG molecules *in vitro*. **A**, RMA-T cells bearing bio-B7-IgG prime specific CD8⁺ CTLs *in vitro*. Nonreplicating RMA-T cells were pulsed with bio-B7-IgG molecules as described in "Materials and Methods" and incubated with purified naive CD8⁺ T cells. T-cell blasts were assayed 6 days later for cytolytic activity using Cr⁵¹-labeled nontransfected RMA. Nonspecific killing was measured using RMA-S cells as targets and did not exceed that induced by RMA cells bearing bio-IgG. **B**, molecular requirements for *in vitro* priming of naive CD8⁺ T cells by RMA-T cells bearing bio-B7-2-IgG. Purified CTLA4-Ig, anti-D^b mAb, or anti-CD8 mAb was added to the priming cultures described above. Cytolytic activity of T-cell blasts was assayed after 6 days of culture. Nonspecific killing of RMA-S targets was 5% at a 100:1 E:T ratio. **C**, adoptive immunotherapy with tumor-specific CD8⁺ CTLs expanded *in vitro* by RMA-T cells bearing bio-B7-IgG molecules. CTLs were restimulated 10 days after *in vitro* priming using RMA-T bearing bio-B7-2-IgG cells. After 8 days, 1×10^6 CTLs, together with IL-2, were injected i.p. into every mouse bearing a 72-h-old RMA tumor as described in "Materials and Methods." Alloreactive CTLs were induced and expanded *in vitro* using nonreplicating BALB/c-derived (H-2^d) J558L plasmacytoma cells. Mice that remained tumor free 30 days after CTL transfer were rechallenged contralaterally with 10^5 living RMA cells and assessed for the development of a protective response. **D**, nonreplicating RMA-T cells bearing bio-B7-IgG molecules are efficient therapeutic vaccines in tumor-bearing mice. One million nonreplicating RMA-T cells bearing bio-B7-2-IgG were injected s.c. in the right flank of mice bearing a 72-h-old tumor generated by injecting 3×10^4 RMA cells s.c. into the left flank. The therapeutic regimen consisted of two injections per week for 3 weeks. Mice that remained tumor free for 25 days were rechallenged contralaterally with 10^5 living RMA cells and assessed for the development of a protective response.

also efficiently expand tumor-specific CTLs *in vitro*, suitable for adoptive immunotherapy. Ten days after *in vitro* priming, CTLs were restimulated with RMA-T cells coated with bio-B7-IgG molecules and allowed to expand for an additional 8 days in the presence of IL-2. CTLs underwent a 3-fold expansion when restimulated by RMA-T cells coated with bio-B7-IgG and not when restimulated with bio-IgG. At day 8 after restimulation, 10^6 CTLs were transferred into every mouse bearing a 72-h-old RMA tumor generated by the s.c. injection of 3×10^4 living cells. As shown in Fig. 1C, all 20 mice receiving the adoptive transfer of anti-RMA CTLs rejected the primary tumor. Moreover, 80% of the cured mice rejected a subsequent contralateral challenge with 1×10^5 living RMA cells, indicating that a systemic memory response was induced (Fig. 1C).

Active Immunotherapy with Nonreplicating RMA-T Cells Bearing bio-B7-Ig. As a second therapeutic approach based on the *in vitro* targeting of bio-B7-IgG molecules, we evaluated the use of nonreplicating RMA-T cells coated with bio-B7-IgG as whole cell vaccines. One million nonreplicating RMA-T cells coated with bio-B7-2-IgG were injected s.c. into syngeneic C57BL/6 mice bearing a 3-day-old contralateral s.c. RMA tumor. As shown in Fig. 1D, 60% of the mice were cured by this treatment. All of the cured mice developed a memory response, as indicated by the rejection of a subsequent contralateral challenge with 10^5 living parental RMA cells. Nonreplicating tumor cells bearing soluble bio-B7-IgG on their surface can therefore function as a whole cell vaccine for active immunotherapy.

Immunotherapy by Targeting Established RMA-T Tumors with bio-B7-IgG. The therapeutic efficacy of targeting bio-B7-IgG to tumors by the three-step approach was evaluated *in vivo*. In these

experiments, all of the targeting compounds (antibody, avidin, and bio-B7-IgG) were administered i.p. The administration of avidin 24 h after the injection of anti-Thy 1.1 biotinylated mAb causes rapid clearance of the antibody from the circulation, as determined by ELISA on the sera of treated animals (data not shown). Moreover, cytofluorimetric analysis of *ex vivo* lymphoma cells showed that bio-B7-2-IgG was still present on the tumor 24 h after complete treatment, but not when avidin was omitted (Fig. 2A). These results show that tumor pretargeting with antibody/avidin complexes allows specific and persistent homing of bio-B7-IgG on tumors.

To assess the therapeutic efficacy of the three-step targeting regimen *in vivo*, mice underwent the treatment at different times after receiving a s.c. inoculum of 3×10^4 living RMA-T cells. As shown in Fig. 2B, when the treatment started 1 day after the tumor inoculum, 40% and 60% of the mice treated with bio-B7-1-IgG and bio-B7-2-IgG, respectively, rejected the primary tumor. Tumor rejection required the administration of all of the components of the three-step approach because omitting either biotin-anti-Thy 1.1 mAb or avidin or both before injecting bio-B7-2-IgG did not result in any therapeutic effects, suggesting that bio-B7-IgG molecules ought to be targeted onto the tumor (data not shown). All of the cured mice also rejected a secondary contralateral challenge with parental RMA cells, indicating that a long-lasting protective immunity was induced (data not shown). When the three-step-mediated treatment with bio-B7-2-IgG was carried out 3 days after the primary tumor inoculum, the mice developed a tumor, although there was a significantly delay (Fig. 2C). Thus, in the case of RMA lymphoma, the therapeutic response appears to be dependent on the tumor burden.

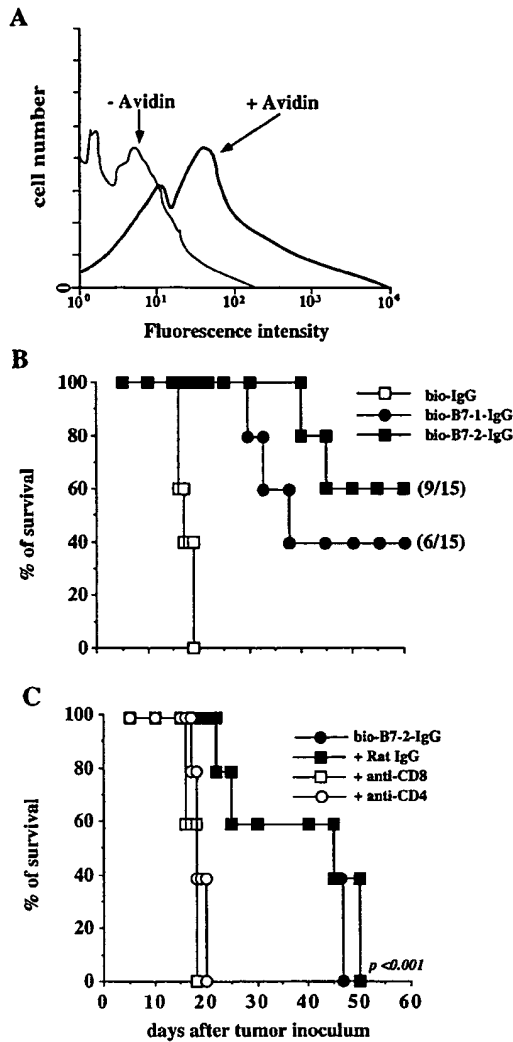


Fig. 2. Therapeutic effects of the *in vivo* targeting of RMA tumor cells with bio-B7-IgG molecules. **A**, efficacy of *in vivo* tumor targeting with bio-B7-IgG by the three-step strategy. One million living RMA-T cells were injected s.c. into B6 mice, and 24 h later, the mice were injected i.p. with 50 μ g of bio-19E12 mAb [F(ab')₂], followed 18 h later by 50 μ g of NAV, and 6 h later by 50 μ g of bio-B7-2-IgG. Animals were killed 24 h after bio-B7-2-IgG injection to excise the tumor mass, which was minced to a single cell suspension and immediately analyzed by flow cytometry for the presence of B7-2-IgG molecules bound to the tumor cells. Histograms indicating the amount of human B7-IgG molecules targeted to tumor cells with or without NAV are shown. One of two reproducible experiments is shown. **B**, treatment of RMA-T tumors by *in vivo* targeting with bio-B7-IgG. Mice were challenged s.c. with 3×10^4 living RMA-T cells, followed 24 h later by the i.p. injection of bio-19E12 anti-Thy 1.1 mAb [F(ab')₂; first step]. NAV was injected 24 h after bio-19E12 (second step), followed 6 h later by either bio-B7-1-IgG or bio-B7-2-IgG (third step). Mice were then scored for tumor growth and sacrificed when the mean tumor diameter reached 10 mm. **C**, both CD4⁺ and CD8⁺ T cells play a critical role in the antitumor response induced by *in vivo* targeted bio-B7-2-IgG. Two days and 1 day before the s.c. injection of 3×10^4 living RMA-T cells, mice were treated i.p. with either control rat IgG, anti-CD4, or anti-CD8 mAb. Injection of the mAbs continued once a week throughout the experiment. The three-step protocol started 72 h after the tumor inoculum. Mice were scored for tumor growth and sacrificed when the mean tumor diameter reached 10 mm. The survival distribution curves of mice receiving only control bio-IgG or bio-B7-2-IgG plus either depleting mAb were comparable. *P* refers to the increased survival of mice treated with only bio-B7-2-IgG compared to that of mice treated with bio-B7-2-IgG and anti-CD8 or anti-CD4 mAb.

Tumor Targeting with bio-B7-IgG Molecules Enhances Both Expansion in the Spleen and Mobilization into the Tumor of CD8⁺ T Cells. To determine the contribution of T cells to the antitumor response induced by *in vivo* tumor targeting with bio-B7-IgG, either CD8⁺ or CD4⁺ T cells were depleted from mice before starting the three-step treatment. As shown in Fig. 2C, depletion of either subset abolished the therapeutic effect of bio-B7-2-IgG on

RMA tumors, indicating that both CD4⁺ and CD8⁺ T cells are critically involved in the antitumor response induced by targeting. Mice undergoing *in vivo* tumor targeting with bio-B7-IgG were also evaluated for the development of a tumor-specific CTL response. As shown in Fig. 3A, only mice that received bio-B7-2-IgG developed a consistent RMA-specific CTL response after the *in vitro* restimulation of splenic T cells. Of note, both mice from the bio-B7-IgG-treated group did not show a measurable s.c. tumor when sacrificed, whereas both mice from the bio-IgG-treated group displayed macroscopic s.c. tumors (mouse 1 had a larger tumor than mouse 2).

Because the Rauscher virus-derived gag-Leader immunodominant epitope presented by RMA cells on D^b is mainly recognized by CD8⁺ CTLs expressing the TCR V β 5.2 family (17, 18), we also investigated whether the *in vivo* targeting of RMA-T tumor with biotin-B7-2-Ig caused a sizeable expansion of primed CD8⁺ V β 5⁺ T cells. *In vivo* targeting of bio-B7-IgG caused a 2- to 3-fold expansion of CD8⁺ TCR V β 5⁺ splenic T cells expressing the antigen-primed markers CD44^{high} (Fig. 3B) or CD62L^{low} (data not shown). No significant expansions within CD8⁺ CD44^{high} T cells expressing control TCR V β 6, V β 8, and V β 13 was observed, indicating that CD8⁺ T cells expressing TCR V β 5 were selectively expanded (data not shown).

We finally determined whether *in vivo* targeting of tumor with

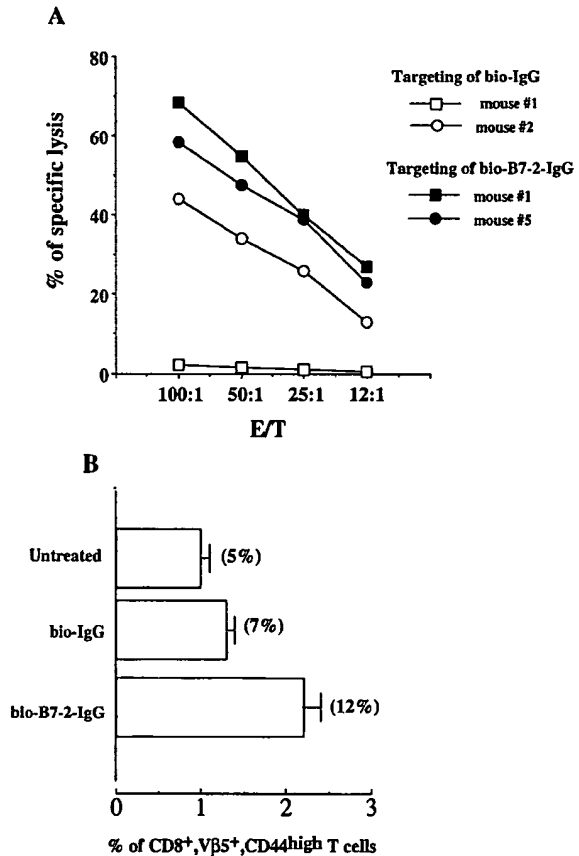


Fig. 3. *In vivo* targeting of RMA-T cells with bio-B7-2-IgG expands RMA-specific CTLs. **A**, induction of RMA-specific CTLs by the *in vivo* targeting strategy. Mice bearing a 24-h-old RMA tumor were subjected to the bio-B7-IgG or bio-IgG targeting protocol. After 10 days, splenic T cells were purified from two mice per experimental condition and restimulated *in vitro* with parental RMA cells. The cytolytic activity of these CTLs was determined 6 days later against ⁵¹Cr-labeled RMA cells. **B**, *in vivo* tumor targeting with bio-B7-2-IgG expands RMA-specific CD8⁺ V β 5⁺ CD44^{high} T cells. Spleen cells obtained from mice 10 days after starting the targeting protocol described in **B** were stained with anti-CD8, anti-V β 5, and anti-CD44 mAb and analyzed by fluorescence-activated cell sorting. The percentage of V β 5⁺ CD44^{high} CD8⁺ T cells in naive, bio-IgG-, and bio-B7-2-IgG-treated mice is shown in parentheses. Three mice per group were analyzed.

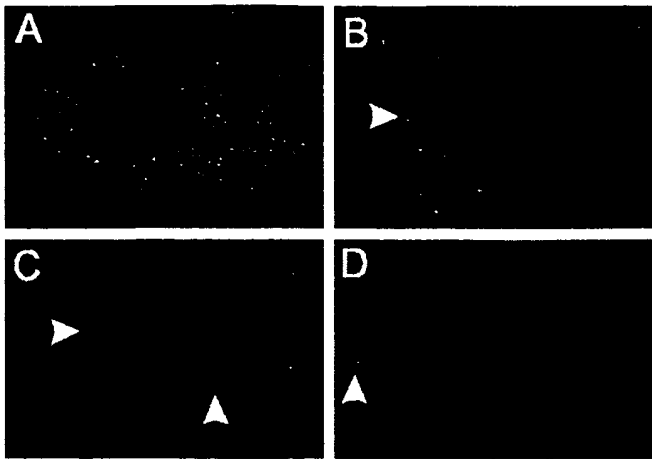


Fig. 4. Tumor targeting with bio-B7-IgG enhances intratumor infiltration by CD8⁺ T cells. Mice bearing a 24-h-old RMA-T tumor underwent the three-step tumor targeting procedure with bio-B7-2-IgG (A and C) or bio-IgG (B and D). After 12 days (corresponding to 15 from the tumor inoculum), tumors were excised, cut in sections (10 μ m), and stained with FITC-conjugated anti-CD8 (A and B) or anti-CD4 (C and D) mAbs. Arrows indicate representative positive T cells.

bio-B7-IgG enhanced intratumor infiltration by T cells. The immunohistochemical analysis of RMA-T tumors excised 13 days after injecting bio-B7-IgG into mice revealed the presence of a marked CD8⁺ T cells and, although to a lower extent, CD4⁺ T cell infiltration (Fig. 4, A and C). The control treatment with bio-IgG did not significantly enhance intratumor infiltration by CD8⁺ (Fig. 4B) and CD4⁺ T cells (Fig. 4D), giving a picture comparable to that of untreated wild-type RMA tumors (data not shown). Collectively, these results indicate that *in vivo* tumor targeting with soluble bio-B7-IgG costimulatory molecules enhances the endogenous antitumor immunity by determining a selective expansion of tumor-specific CTLs in the spleen as well as by enhancing T-cell infiltration into the tumor.

bio-B7-1-IgG but not bio-B7-2-IgG Is Effective when the Targeting Is Performed on Poorly Immunogenic TS/A Mammary Adenocarcinoma. We determined the efficacy of targeting bio-B7-IgG molecules on a second tumor, the poorly immunogenic TS/A mammary adenocarcinoma. At variance with RMA-T cells, TS/A-T cells coated with bio-B7-1-IgG, but not with bio-B7-2-IgG, primed specific CTLs *in vitro* (Fig. 5A). In these experiments, TS/A-T cells were covered with a comparable amount of each of the bio-B7-IgG molecules, as determined by cytofluorimetric analysis (data not shown). In keeping with the *in vitro* data, targeting established TS/A-T adenocarcinoma *in vivo* with bio-B7-1-IgG, but not with bio-B7-2-IgG, induced a 10% tumor rejection with a significant overall delay in the tumor growth (Fig. 5B). Thus, at variance with the results obtained with the RMA model, only bio-B7-1-IgG induced an immune response when three-step-mediated targeting was performed with the TS/A mammary adenocarcinoma.

DISCUSSION

We have shown that targeting tumors with recombinant B7-Ig costimulatory molecules by the three-step approach is an efficient strategy to induce and expand therapeutic tumor-specific T cells both *in vitro* and *in vivo*. Targeted soluble bio-B7-IgG and transfected full-length molecules appear to be fully comparable from the functional point of view (11).⁵ However, the three-step procedure may overcome the technical limitations that genetic engineering still shows

in the generation of tumor vaccines expressing B7. The three-step approach is in fact rapid and simple and does not require the generation of cell lines from each tumor. Primary tumor material from surgical specimens could be armed by the three-step method with bio-B7-IgG molecules, irradiated, and injected back into patients within hours. Moreover, we have also demonstrated that direct tumor targeting by systemic injection of biotinylated antibody, avidin, and bio-B7-IgG conjugate induces a therapeutic immunity against established tumors. The immune activation observed with this treatment was likely dependent on the binding of bio-B7-IgG to tumor cells because no response was observed when either biotin-anti-Thy 1.1 or avidin or both were omitted in control experiments. The relevance of this finding relies on the fact that the treatment was carried out entirely *in vivo*, without previous manipulation of tumor cells *in vitro*.

Depletion experiments indicate that both CD4⁺ and CD8⁺ T cells are critically involved in tumor rejection induced by *in vivo* targeted B7-IgG molecules, although the depletion regimen used does not allow us to determine whether either subset is required during the priming and effector phase of the antitumor response. It is clear from this data, however, that targeted B7-IgG cannot bypass the requirement for CD4⁺ T cell help to induce antitumor CD8⁺ CTLs. Because

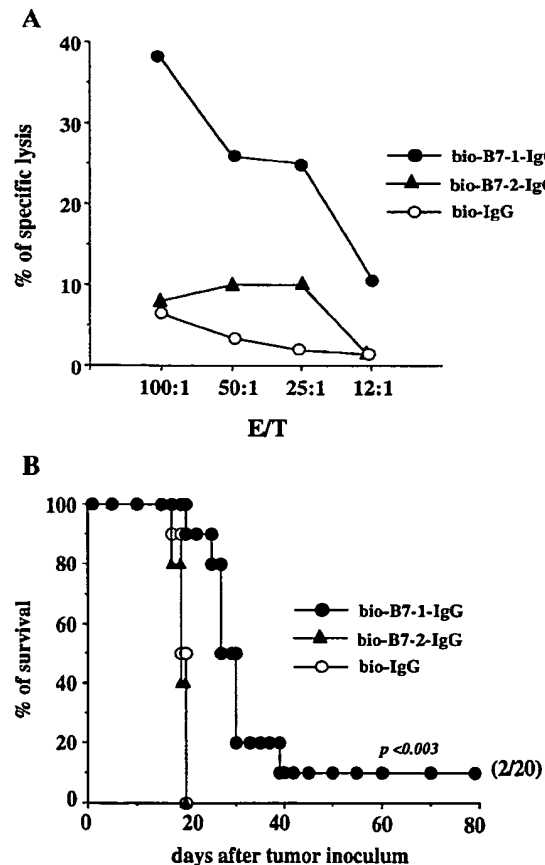


Fig. 5. Bio-B7-1-IgG but not bio-B7-2-IgG is effective when targeting is performed on poorly immunogenic TS/A mammary adenocarcinoma. A, poorly immunogenic TS/A mammary adenocarcinoma bearing bio-B7-1-IgG but not bio-B7-2-IgG prime naive CTLs *in vitro*. Total spleen cells from naive BALB/c mice were cultured with nonreplicating TS/A-T cells bearing bio-B7-1-IgG, bio-B7-2-IgG, or bio-IgG, as described in "Materials and Methods." Cytotoxic activity was measured after 6 days. Nonspecific killing measured on the RMA-S target cells was comparable to that induced by TS/A-T cells bearing bio-IgG. One representative experiment of three experiments is shown. B, therapeutic efficacy of the three-step-dependent *in vivo* targeting of TS/A-T tumors with bio-B7-IgG. The three-step protocol started 48 h after the s.c. inoculum of 4×10^4 living TS/A-T cells. Mice were scored for tumor growth and sacrificed when the mean tumor diameter reached 10 mm. *P* refers to the increased survival of mice treated with bio-B7-1-IgG compared to that of mice treated with either bio-B7-2-IgG or bio-IgG.

⁵ A. Martin-Fontecha, unpublished results.

the RMA tumor used in this experiment cannot express MHC class II molecules, this result suggests an important role for endogenous MHC class II⁺ APCs in capturing, processing, and representing TAAs to CD4⁺ T cells. Whether bio-B7-IgG molecules enhance the cross-priming of tumor-specific CD4⁺ and CD8⁺ T cells by endogenous APC or allow for direct activation of tumor-specific CD8⁺ T cells by targeted tumor cells remains to be established. Targeted bio-B7-IgG most likely enhances both direct presentation and cross-priming of TAA, as recently shown for the full-length B7-1 molecule transfected into TS/A adenocarcinoma (19).

RMA tumor cells are intrinsically immunogenic (10), therefore it is not surprising that they can induce tumor-specific CTLs in some animals in the absence of targeted bio-B7-IgG. However, these T cells are mostly confined in the secondary lymphoid organs and do not efficiently infiltrate the tumor. Accordingly, the CTLs induced by parental RMA tumors do not have therapeutic efficacy. In contrast, targeting RMA tumors *in vivo* with bio-B7-IgG results in two major effects on the host's immune system: (a) it markedly enhances the expansion of RMA-specific CD8⁺ Vβ5⁺ CTLs in the spleen of all of the mice tested; and (b) it induces the mobilization of CD8⁺ into the tumor and, less prominently, the CD4⁺ T cells. Conceivably, these two effects are sequentially related, thus the intratumor infiltration by T cells would reflect the induction of an efficient systemic antitumor immunity by targeted bio-B7-IgG. It is also possible, however, that the targeted costimulatory molecules exert some of their effects locally, at the tumor site. For instance, it has been shown that B7/CD28 interaction reduces the apoptosis of T cells during both the induction and effector phases of the immune response (4, 20–22). Thus, targeted B7-IgG may rescue tumor-infiltrating lymphocytes from dying after killing tumor cells, resulting in an improved effector phase at the tumor site. Furthermore, B7-dependent engagement of CD28 at the tumor site may enhance the local production of pro-inflammatory cytokines, favoring the induction of an inflammatory reaction critical for the progression of the antitumor response.

The antitumor mechanisms triggered by the B7 targeting were sufficient to control the growth of only relatively small RMA tumors, suggesting that tumor targeting with soluble B7-IgG molecules would therefore be better aimed at the treatment of minimal residual tumor disease. One possible explanation for this phenomenon is that in the case of large tumors, the CTL expansion is still insufficient to achieve the proper CTL:tumor cell ratio necessary to efficiently kill all of the neoplastic cells. Alternatively, the antitumor response is suppressed progressively in the host upon RMA growth *in vivo*. However, experiments performed with immunogenic tumors showing the presence of an intact immune system in mice bearing a large tumor burden do not support this hypothesis (23).

Whereas both B7-IgG molecules induce efficient antitumor responses when targeted on RMA cells both *in vitro* and *in vivo*, only B7-1-IgG works with TS/A cells under the same conditions. The different effect of B7-1 and B7-2 on the two tumor cell lines is intriguing. We can exclude that it is due to particular features of the soluble chimeric molecules because the B7-IgG molecules used with RMA and TS/A tumors were derived from the same batches. Moreover, the *in vitro* priming data were obtained using either RMA-T or TS/A-T cells covered with a comparable amount of each bio-B7-IgG molecule, thus excluding major quantitative differences between the two soluble costimulatory molecules during priming. Hence, it is tempting to speculate that intrinsic biological differences between B7-1 and B7-2 may explain their unequal effects on RMA and TS/A cells. Several studies performed with other tumor models have underscored the nonredundancy of B7-1- and B7-2-dependent costimulation, usually revealing the superior efficacy of B7-1 (24–26). These studies, together with our results, would suggest the use of B7-1 as a

universal costimulatory molecule, although B7-2 could be more efficient with some tumors.

The three-step-dependent targeting strategy used in this study offers several alternative advantages over the use of conventional immunoconjugates: (a) rapid circulation clearance of the biotinylated mAb by avidin injected as the second step, which improves tumor:background ratios (27, 28); (b) modularity, which allows the use of differential doses of targeting and effector components; and (c) an easy combination of different biotinylated antitumor mAbs as well as biotinylated effector molecules, avoiding the requirement to generate all of the corresponding bispecific molecules, each carrying the desired tumor specificity and effector arm. In this respect, a number of mAbs specific for different surface molecules whose expression is restricted to tumors are already available for clinical applications and could be used to target soluble B7 costimulatory molecules *in vivo* (29–31). Moreover, the feasibility of tumor avidination in patients is well documented, and it is currently used in nuclear medicine to increase the uptake and localization of radioactive-labeled biotin for either diagnostic or therapeutic purposes (27, 28, 32–36).

Tumor pretargeting with avidin can also be exploited to deliver other biotin-conjugated effectors to the tumor site. In particular, we found that the pretargeting system can increase the amount and the persistence of biotin-TNF on tumor cells and that cell-bound TNF can trigger antitumor responses *in vivo* (15, 37). Experiments are under way to assess whether a combination of *in vivo* targeting of soluble recombinant B7 and cytokines results in a stronger antitumor response. Moreover, it will also be interesting to determine whether the association between active vaccination with TAA and *in vivo* tumor targeting with B7-IgG can improve the antitumor response.

Collectively, the results presented in this study suggest that tumor targeting with soluble recombinant B7 costimulatory molecules may represent a promising strategy to enhance the host's antitumor immune response.

ACKNOWLEDGMENTS

We thank Dr. U. Pessara and P. Protti for criticisms and Prof. A. Siccaldi for advice on the use of the three-step targeting method.

REFERENCES

- Boon, T., Cerottini, J. C., Van den Eynde, B., van der Bruggen, P., and Van Pel, A. Tumor antigens recognized by T lymphocytes. *Annu. Rev. Immunol.*, 12: 337–365, 1994.
- Schwartz, R. H. Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. *Cell*, 71: 1065–1068, 1992.
- June, C. H., Bluestone, J. A., Nadler, L. M., and Thompson, C. B. The B7 and CD28 receptor families. *Immunol. Today*, 15: 321–331, 1994.
- Lenschow, D. J., Walunas, T. L., and Bluestone, J. A. CD28/B7 system of T cell costimulation. *Annu. Rev. Immunol.*, 14: 233–258, 1996.
- Chen, L., Ashe, S., Brady, W. A., Hellstrom, I., Hellstrom, K. E., Ledbetter, J. A., McGowan, P., and Linsley, P. S. Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell*, 71: 1093–1102, 1992.
- Chen, L., Linsley, P. S., and Hellstrom, K. E. Costimulation of T cells for tumor immunity. *Immunol. Today*, 14: 483–486, 1993.
- Townsend, S. E., and Allison, J. P. Tumor rejection after direct costimulation of CD8⁺ T cells by B7-transfected melanoma cells. *Science (Washington DC)*, 259: 368–370, 1993.
- Hellstrom, K. E., Hellstrom, I., and Chen, L. Can co-stimulated tumor immunity be therapeutically efficacious? *Immunol. Rev.*, 145: 123–145, 1995.
- Yang, G., Hellstrom, K. E., Hellstrom, I., and Chen, L. Antitumor immunity elicited by tumor cells transfected with B7-2, a second ligand for CD28/CTLA-4 costimulatory molecules. *J. Immunol.*, 154: 2794–2800, 1995.
- Martin-Fontecha, A., Cavallo, F., Bellone, M., Heltai, S., Iezzi, G., Tornaghi, P., Nabavi, N., Forni, G., Dellabona, P., and Casorati, G. Heterogeneous effects of B7-1 and B7-2 in the induction of both protective and therapeutic antitumor immunity against different mouse tumors. *Eur. J. Immunol.*, 26: 1851–1859, 1996.
- Cavallo, F., Martin-Fontecha, A., Bellone, M., Heltai, S., Gatti, E., Tornaghi, P., Freschi, M., Forni, G., Dellabona, P., and Casorati, G. Co-expression of B7-1 and

- ICAM-1 on tumors is required for rejection and the establishment of a memory response. *Eur. J. Immunol.*, 25: 1154–1162, 1995.
12. Geldhof, A. B., Raes, G., Bakkus, M., Devos, S., Thielemans, K., and De Baetselier, P. Expression of B7-1 by highly metastatic mouse T lymphomas induces optimal natural killer cell-mediated cytotoxicity. *Cancer Res.*, 55: 2730–2733, 1995.
 13. Wu, T. C., Huang, A. Y., Jaffee, E. M., Levitsky, H. I., and Pardoll, D. M. A reassessment of the role of B7-1 expression in tumor rejection. *J. Exp. Med.*, 182: 1415–1421, 1995.
 14. Chambers, B. J., Salcedo, M., and Ljunggren, H. G. Triggering of natural killer cells by the costimulatory molecule CD80 (B7-1). *Immunity*, 5: 311–317, 1996.
 15. Moro, M., Pelagi, M., Fulci, G., Paganelli, G., Dellabona, P., Casorati, G., Siccaldi, A. G., and Corti, A. Tumor cell targeting with antibody-avidin complexes and biotinylated tumor necrosis factor α . *Cancer Res.*, 57: 1922–1928, 1997.
 16. Trautnecker, A., Lanzavecchia, A., and Karjalainen, K. Bispecific single chain molecules (Janusins) target cytotoxic lymphocytes on HIV-infected cells. *EMBO J.*, 10: 3655–3659, 1991.
 17. Iezzi, G., Rivolta, L., Ronchetti, A., Martin-Fontecha, A., Rosato, A., Protti, M. P., Sabbadini, M. G., and Bellone, M. The immunogenicity of experimental tumors is strongly biased by the expression of dominant viral cytotoxic T-lymphocyte epitopes. *Cancer Res.*, 57: 2564–2568, 1997.
 18. Brawand, P., Biasi, G., Horvath, C., Cerottini, J. C., and MacDonald, H. R. Flow-microfluorometric monitoring of oligoclonal CD8+ T cell responses to an immunodominant Moloney leukemia virus-encoded epitope *in vivo*. *J. Immunol.*, 160: 1659–1665, 1998.
 19. Cayeux, S., Richter, G., Noffz, G., Dorken, B., and Blankenstein, T. Influence of gene-modified (IL-7, IL-4, and B7) tumor cell vaccines on tumor antigen presentation. *J. Immunol.*, 158: 2834–2841, 1997.
 20. Boise, L. H., Minn, A. J., Noel, P. J., June, C. H., Accavitti, M. A., Lindsten, T., and Thompson, C. B. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-X_L. *Immunity*, 3: 87–98, 1995.
 21. Van Parijs, L., Ibraghimov, A., and Abbas, A. K. The roles of costimulation and Fas in T cell apoptosis and peripheral tolerance. *Immunity*, 4: 321–328, 1996.
 22. Daniel, P. T., Kroidl, A., Cayeux, S., Bargou, R., Blankenstein, T., and Dorken, B. Costimulatory signals through B7.1/CD28 prevent T cell apoptosis during target cell lysis. *J. Immunol.*, 159: 3808–3815, 1997.
 23. Chen, L. P. Immunological ignorance of silent antigens as an explanation of tumor evasion. *Immunol. Today*, 19: 27–30, 1998.
 24. Gajewski, T. F. B7-1 but not B7-2 efficiently costimulates CD8+ T lymphocytes in the P815 tumor system *in vitro*. *J. Immunol.*, 156: 465–472, 1996.
 25. Leong, C. C., Marley, J. V., Loh, S., Milech, N., Robinson, B. W., and Garlepp, M. J. Transfection of the gene for B7-1 but not B7-2 can induce immunity to murine malignant mesothelioma. *Int. J. Cancer*, 71: 476–482, 1997.
 26. Matulonis, U., Dosiou, C., Freeman, G., Lamont, C., Mauch, P., Nadler, L. M., and Griffin, J. D. B7-1 is superior to B7-2 costimulation in the induction and maintenance of T cell-mediated antileukemia immunity. Further evidence that B7-1 and B7-2 are functionally distinct. *J. Immunol.*, 156: 1126–1131, 1996.
 27. Stella, M., De Nardi, P., Paganelli, G., Magnani, P., Mangili, F., Sassi, I., Baratti, D., Gini, P., Zito, F., Cristallo, M., Fazio, F., and DiCarlo, V. Avidin-biotin system in radioimmunoguided surgery for colorectal cancer. Advantages and limits. *Dis. Colon Rectum*, 37: 335–343, 1994.
 28. Yao, Z., Zhang, M., Kobayashi, H., Sakahara, H., Nakada, H., Yamashina, I., and Konishi, J. Improved targeting of radiolabeled streptavidin in tumors pretargeted with biotinylated monoclonal antibodies through an avidin chase. *J. Nucl. Med.*, 36: 837–841, 1995.
 29. Goldenberg, D. M., Larson, S. M., Reisfeld, R. A., and Schlom, J. Targeting cancer with radiolabeled antibodies. *Immunol. Today*, 16: 261–264, 1995.
 30. Uhr, J. W., Scheuermann, R. H., Street, N. E., and Vitetta, E. S. Cancer dormancy: opportunities for new therapeutic approaches. *Nat. Med.*, 3: 505–509, 1997.
 31. Scott, A. M., and Welt, S. Antibody-based immunological therapies. *Curr. Opin. Immunol.*, 9: 717–722, 1997.
 32. Paganelli, G., Magnani, P., and Fazio, F. Pretargeting of carcinomas with the avidin-biotin system. *Int. J. Biol. Markers*, 8: 155–159, 1993.
 33. Saga, T., Weinstein, J. N., Jeong, J. M., Heya, T., Lee, J. T., Le, N., Paik, C. H., Sung, C., and Neumann, R. D. Two-step targeting of experimental lung metastases with biotinylated antibody and radiolabeled streptavidin. *Cancer Res.*, 54: 2160–2165, 1994.
 34. Karacay, H., Sharkey, R. M., Govindan, S. V., McBride, W. J., Goldenberg, D. M., Hansen, H. J., and Griffiths, G. L. Development of a streptavidin-anti-carcinoembryonic antigen antibody, radiolabeled biotin pretargeting method for radioimmunotherapy of colorectal cancer. Reagent development. *Bioconjug. Chem.*, 8: 585–594, 1997.
 35. Sharkey, R. M., Karacay, H., Griffiths, G. L., Behr, T. M., Blumenthal, R. D., Mattes, M. J., Hansen, H. J., and Goldenberg, D. M. Development of a streptavidin-anti-carcinoembryonic antigen antibody, radiolabeled biotin pretargeting method for radioimmunotherapy of colorectal cancer. Studies in a human colon cancer xenograft model. *Bioconjug. Chem.*, 8: 595–604, 1997.
 36. Zhang, M. L., Yao, Z. S., Sakahara, H., Saga, T., Nakamoto, Y., Sato, N., Zhao, S. J., Nakada, H., Yamashina, I., and Konishi, J. Effect Of administration route and dose of streptavidin or biotin on the tumor uptake of radioactivity in intraperitoneal tumor with multistep targeting. *Nucl. Med. Biol.*, 25: 101–105, 1998.
 37. Gasparri, A., Moro, M., Cumis, F., Sacchi, A., Pagano, S., Veglia, F., Casoratio, G., Siccaldi, A. G., Dellabona, P., and Corti, A. Tumor pre-targeting with avidin improves the therapeutic index of biotinylated tumor necrosis factor α in a mouse model. *Cancer Res.*, in press, 1999.